

1996

Immobilization of *P. Digitatum* and Bioconversion of Limonene to Alpha-Terpineol.

Qiang Tan

Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Recommended Citation

Tan, Qiang, "Immobilization of *P. Digitatum* and Bioconversion of Limonene to Alpha-Terpineol." (1996). *LSU Historical Dissertations and Theses*. 6312.

https://digitalcommons.lsu.edu/gradschool_disstheses/6312

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Historical Dissertations and Theses by an authorized administrator of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

IMMOBILIZATION OF *P.DIGITATUM* AND BIOCONVERSION OF
LIMONENE TO ALPHA-TERPINEOL

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Food Science

by

Qiang Tan

B.S., Southwest Agricultural University, P. R. China. 1984

M.S. Louisiana State University, Baton Rouge. 1992

December. 1996

UMI Number: 9712860

UMI Microform 9712860
Copyright 1997, by UMI Company. All rights reserved.

**This microform edition is protected against unauthorized
copying under Title 17, United States Code.**

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

ACKNOWLEDGMENT

The author wishes to express his sincere and deepest appreciation to his present advisor, Dr. D. Day, for his continued support throughout the project his insightful advice as well as his encouragement and patience throughout the years. Appreciation is also extended to his former advisor, Dr. Keith R. Cadwallader, for his help in initiating this research topic and tremendous technical support during his tenure in the Food Science Department.

Appreciation is expressed to Dr. Auttis Mullins, former Head, and Dr. Douglas L. Park, current Head, Department of Food Science, for their support of this research. Appreciation is extended to the Audubon Sugar Institute for providing the facilities and chemicals to conduct this research. Sincere thanks are given to Drs. Robert M. Grodner, Paul W. Wilson, Joseph A. Liuzzo, and E. William Wischusen (Dean's Representative) for serving on the dissertation committee and for their valuable assistance and advice.

The author would like to thank his lab colleges, Ms. D. Sarka, Mr. M. Ott, Mr. Sunkyun Yoo, Dr. Hau Yin Chung, and Ms. Narumol Thongwai for valuable discussions.

Finally the author dedicates this work to his wife, Yu Yang, his daughter Jacqueline S. Tan and his families. Their encouragement, love, patience and understanding have made this work possible.

TABLE OF CONTENTS

ACKNOWLEDGMENT.....	ii
LIST OF TABLES	vi
LIST OF FIGURES.....	vii
ABSTRACT.....	x
INTRODUCTION.....	1
REVIEW OF LITERATURE	3
I. Terpenes and terpinoids	3
1. Background	3
2. Classification.....	4
3. Industrial uses of terpenes.....	7
4. Monoterpenes.....	7
5. Limonene	10
6. α -Terpineol	11
II. Microbial bioconversion of monoterpenes	12
1. Definition of bioconversion or biotransformation	12
2. Methods for bioconversion	13
3. Bacterial and fungal bioconversion of monoterpenes.....	15
III. Microbial systems for hydroxylation reactions.....	27
1. Microbiological hydroxylations	27
2. Microbial hydroxylation of aliphatic and aromatic compounds	27
3. Microbial hydroxylation of terpinoids	30
4. Enzymatic systems and mechanisms related to microbial hydroxylation.....	31
5. Stereochemistry of microbial hydroxylation.....	39
IV. Fungal immobilization.....	41
V. Whole-cell bioconversions in aqueous-organic solvent systems.....	44
1. Use of organic solvents in whole-cell biotransformations.....	44
2. Solvent selection for whole cell biotransformations.....	45
3. Biotransformations on aqueous two-phase systems.....	49
VI. This study.....	51
MATERIALS AND METHODS.....	52
I. Chemicals.....	52
II. Organism and growth conditions	52
III. Growth and bioconversion activity	53

IV.	Analytical methods	54
V.	Extraction recovery	56
VI.	Substrate induction.....	57
VII.	Enzyme inhibition.....	57
VIII.	Determination of the Michaelis-Menten constant.....	58
IX.	Determination of product stereochemistry.....	58
X.	Immobilization of <i>P. digitatum</i> on calcium alginate.....	59
XI.	Separation of the calcium alginate beads from cell mass	60
XII.	Calcium alginate beads storage stability	60
XIII.	Optimization of limonene bioconversion.....	60
	1. Aeration.....	60
	2. Temperature	61
	3. pH.....	61
	4. Limonene concentration.....	62
	5. Contact time	62
XIV.	Bioconversion of immobilized cells with batch and repeated batch form.....	62
XV.	Bioconversion of immobilized cells in a continuously fed air-lift bioreactor	63
XVI.	Bioconversion in organic solvent systems	64
	1. Effect of organic solvents on bioconversion of limonene.....	64
	2. Optimization of dioctylphthalate and methanol concentration for free cell bioconversion.....	65
	3. Optimization of Tween 80 concentration for immobilized cell bioconversion.....	65
RESULTS		67
I.	Proof of bioconversion.....	67
II.	Physiological studies.....	67
	1. Growing cells and α -terpineol production	67
	2. Resting cells and α -terpineol production	73
	3. Substrate induction.....	77
	4. End product inhibition	81
	5. Inhibitor studies	81
	6. K_m (app.) of bioconversion of limonene with free and immobilized cells.....	84
	7. Stereochemical properties	84
III.	Optimization of bioconversion conditions.....	87
	1. Aeration.....	87
	2. Temperature	90
	3. pH.....	90
	4. Substrate concentration.....	90
	5. Reaction time	93

IV.	Bioconversion of limonene with immobilized fungal cells	96
1.	Stability of immobilized beads	96
2.	Batch and repeated batch bioconversions	96
3.	Continuous bioreactor	99
V.	Bioconversion of limonene in organic solvent systems	101
DISCUSSION		115
SUMMARY		127
REFERENCES		128
VITA		142

LIST OF TABLES

Table 1.	Organoleptic properties of terpenes	8
Table 2.	Structural categories for monoterpenes.....	9
Table 3.	Advantages and disadvantages of using isolated enzymes and whole cell systems.....	14
Table 4.	Potential advantages of the use of organic solvents in whole-cell biotransformations	46
Table 5.	Potential disadvantages of the use of organic solvents in whole-cell biotransformations	47
Table 6.	Recovery percentage of extract method.....	68
Table 7.	Growth media evaluation for the growth of <i>P. digitatum</i>	72
Table 8.	Bioconversion activity of batch and repeated batch form.....	98
Table 9.	Effect of addition of organic solvents and surfactants as co-solvents of substrate on immobilized beads for the bioconversion of limonene to α -terpineol.....	105
Table 10.	Effect of addition of organic solvents and surfactants as co-solvents of substrate on growing cells for the bioconversion of limonene to α -terpineol.....	106

LIST OF FIGURES

Figure 1.	Ionic scheme for the formation of monoterpenes via the α -terpinyl cation (a) and the terpinen-4-yl cation (b).....	6
Figure 2.	Standard procedures for bioconversion with fungi	16
Figure 3.	Pathways of degradation of limonene	18
Figure 4.	Pathways of microbial degradation of (R)-(+)-limonene	19
Figure 5.	Proposed routes for benzylic hydroxylation.....	36
Figure 6.	Catalytic cycle of cytochrome P-450 dependent mono-oxygenases.....	38
Figure 7.	Total ion chromatogram of <i>P. digitatum</i> bioconversion products extract after 2 days contact with limonene (A). Mass spectrum at retention time 12.52 min (B).....	69
Figure 8.	Total ion chromatogram of standard mixture of α -terpineol and internal standard 1-decanol (A). Mass spectrum at retention time 12.21 min (B)	70
Figure 9.	Total ion chromatogram of extraction from 2 days <i>P. digitatum</i> culture without limonene addition (A). Mass spectrum at retention time 13.53 min (B)	71
Figure 10.	Specific activity of bioconversion by <i>P. digitatum</i> growing cells at 28°C in 24 h	74
Figure 11.	Different aged inocula, effect on growth and bioconversion activity of α -terpineol at 28°C. Assayed after 24 hrs.....	75
Figure 12.	Specific activity of <i>P. digitatum</i> resting cells for bioconversion of limonene	76
Figure 13.	Bioconversion after 200 ppm substrate induction in three different growth stages.....	78
Figure 14.	Comparison of bioconversion activity with 200 ppm substrate added at three points for induction with the control, arrows show the time of induction	79

Figure 15.	Bioconversion extent comparison of induced growing mycelia from early-log phase and non-induced mycelia over a range of substrate concentrations	80
Figure 16.	Effect of α -terpineol concentration on free cell bioconversion of limonene by <i>P. digitatum</i>	82
Figure 17.	Inhibition of phenanthroline on bioconversion of limonene by <i>P. digitatum</i> free and immobilized cells	83
Figure 18.	Lineweaver-Burk plot showing the K_m and V_{max} for limonene bioconversion by growing and immobilized <i>P. digitatum</i> mycelia.....	85
Figure 19.	Total ion chromatograms of bioconversion of (R)-(+)- (A), (S)-(-)- (B), and racemic limonene (C) to α -terpineol by <i>P. digitatum</i>	86
Figure 20.	Oxygen effect on specific activity of free and immobilized cells	88
Figure 21.	Time courses for bioconversion activity of free and immobilized <i>P. digitatum</i> cells assayed under different aeration rates	89
Figure 22.	Temperature optima for bioconversion by <i>P. digitatum</i> with free and immobilized cells	91
Figure 23.	pH optima of bioconversion of limonene by <i>P. digitatum</i> with free and immobilized cells	92
Figure 24.	Effect of substrate concentration on bioconversion activity for free and immobilized <i>P. digitatum</i> mycelia	94
Figure 25.	α -Terpineol production influenced by with substrate contact time for growing cells, resting cells, and immobilized cells.....	95
Figure 26.	Storage stability of immobilized beads for -20°C and 4 °C based on activity.....	97
Figure 27.	Productivity comparison of repeated batch to first batch with immobilized beads. Beads were regenerated after the third batch	100

Figure 28.	Comparison of different aeration rates on α -terpineol productivity relative to control of immobilized cells with flow rate of 0.0072 h^{-1} in an air-lift bioreactor	102
Figure 29.	Comparison of different aeration rates on α -terpineol productivity relative to control of immobilized cells with flow rate of 0.0144 h^{-1} in an air-lift bioreactor	103
Figure 30.	Relative α -terpineol productivity with time at different aeration rates with immobilized cells in a continuous bioreactor.....	104
Figure 31.	Effect of methanol concentration on free cells bioconversion extent and activity relative to the control (100%) without methanol....	108
Figure 32.	Comparison of dioctylphthalate concentration on free cells bioconversion extent and activity relative to the control (100%) without dioctylphthalate.....	109
Figure 33.	Effect of Tween 80 concentration on bioconversion extent and relative activity of immobilized <i>P. digitatum</i> mycelia.....	111
Figure 34.	Time course of α -terpineol production of free <i>P. digitatum</i> mycelia with and without Tween 80 addition.....	112
Figure 35.	Time course of α -terpineol production of immobilized <i>P. digitatum</i> mycelia with and without Tween 80 addition	113

ABSTRACT

The parameters of bioconversion of limonene to α -terpineol by *Penicillium digitatum* were determined. This bioconversion was carried out stereo-specifically and selectively by this fungus. (R)-(+)-limonene was selectively converted to (R)-(+)- α -terpineol by mycelia of this fungus during early growth phase. Immobilization of the fungal mycelia for use in continuous production of this product was possible. Aeration was critical to this bioconversion. The optimum aeration was about 1.6 mg/L for growing cells and immobilized cells. Substrate inhibition of the bioconversion was not observed when the substrate concentration was below 4% (v/v). End product inhibition was observed. The $K_{i_{app}}$ was 12.08 mM. The optimum temperature was 28°C. Optimum pH was 4.5 for resting cells and 7.0 for immobilized cells. The optimum substrate contact time for this bioconversion was 1 to 2 days and 3 to 4 days for free cells and immobilized cells respectively. Several organic solvents and detergents were found to increase bioconversion yield for free and immobilized fungal cells. The half-life of immobilized mycelia was dependent on the amount of aeration. At the 0.3 SLPM aeration, the half-life of immobilized fungal mycelia was about 7 days.

INTRODUCTION

World wide, 90% of the volatile aroma chemicals are synthetics (Layman, 1984). Drawbacks of chemical synthesis include the formation of mixtures of isomers and an increasing preference of the consumer for natural food additives. According to the Code of Federal Regulations (21 CFR 101.22.a.3.), compounds produced or modified by living cells or by their components, including enzymes, may be designated as natural. Furthermore, those products can be considered natural providing that they are derived from natural starting materials. In this respect, biotechnological processes provide many possibilities for production of natural flavors and fragrances.

Biotechnological approaches for synthesis of flavors and fragrances are divisible into two classes according to the production method: microbiological or enzymatic. The microbiological class includes biosynthetic and biotransformation methods. The former refers to the production of chemical compounds by metabolizing cells, whereas the latter is defined as using microbial cells to perform enzymatic modification or interconversion of chemical structures (Welsh, et al., 1989). Previous studies (Schindler and Schmid, 1982, Janssens et al., 1992) have shown that many fungi can catalyze specific conversions of added precursors or intermediates through biotransformation or bioconversion process.

Terpenes are often the major constituents responsible for the characteristic aroma or flavors of essential oils. They are hydrocarbons built from a basic 5-carbon isoprene unit (2-methyl-1,3-butadiene), with structures that may be open chain, cyclic, saturated, or unsaturated. Terpenes and their oxygenated derivatives have been used extensively by the flavor and fragrance industry. According to Welsh, et al. (1989), about 2.8×10^5 kg of

monoterpenes were used in flavor and fragrance industry each year. The usage is increasing. Limonene is an inexpensive terpene compound. The primary sources of limonene are from citrus industry as a by-product. Annual world availability of limonene amounts to about 50 million kg (Braddock and Cadwallader, 1995). Limonene has been used extensively as a starting material in the manufacture of terpene alcohols and ketones (Bauer and Garbe, 1985). α -Terpineol is an important flavor and fragrance chemical because of its lilac like aroma. A common commercial process is hydration of pinene or turpentine oil. The current market price of α -terpineol is about 1.6 fold that of limonene.

Terpenes and their oxygenated derivatives are a favored substrates with which to study bioconversion. About ninety fungal strains are reported to be able to perform bioconversion of terpenes (Janssens et al., 1992). Most of the microbial transformations performed on terpenes have been on monoterpenes (Battacharyya et al., 1960 and 1965, Lanza et al., 1977, Gatfield, 1988). A fungal strain, *P. digitatum*, was found to have the ability to convert limonene to α -terpineol. The objectives of this study were to investigate and optimize conditions of the *P. digitatum* bioconversion and to develop an immobilized fungal system for continuously and efficiently producing α -terpineol.

REVIEW OF LITERATURE

I. Terpenes and terpinoids

1. Background

The biosynthesis of the vast array of natural products produced by both plants and animals occurs by a limited number of biosynthetic pathways. One of the pathways involves the coupling of two or more units of Δ^2 - and Δ^3 -isopentenyl pyrophosphates [Δ^2 and Δ^3 -IPP] by a stereoregulated process, leading to the compounds called terpenes (Erman, 1985). Terpenes are major components of the so called “essential oils” of many flowers, fruits, leaves, and roots of plants. Early studies on the chemical composition of the “essential oils” led to the discovery of a series of related olefins of general formula $C_{10}H_{16}$. These compounds became known as the terpenes, derived from the German terpentine (turpentine). Crystalline, related oxygenated compounds, also with a ten-carbon skeleton, became known as camphors, because of their physical resemblance to true camphor. The early literature talks of “thyme camphor” (thymol) and a “peppermint camphor” (menthol). Later, when structural similarities were classified, the oxygenated compounds were included under the general heading of terpenes, with the term “camphor” being restricted to a specific compound. The term “terpene” is now loosely applied to all compounds with a terpenoid (isoprenoid) structure (Croteau, 1980). The name terpenoid was to be used as the general label for isoprenoids, analogous to the term,

steroid, the name, terpene, should only refer to isoprenoid hydrocarbons. However, both terms now are used interchangeably (Erman, 1985). The terpene class is usually extended to include those compounds which are not naturally occurring, but which are structurally close to natural terpenes (Pinder, 1960).

Terpenes have been found in animals, microorganisms, and some insects. The carotenoids play an important role in plant photosynthesis. Sesquiterpene, a member of the gibberellins, is a plant growth stimulant. Isomeamarone shows potential antimicrobial action. Citronellol protects plants from insect predators (Erman, 1985). Citronellol was also isolated from the scent glands of alligators. Certain insects produce monoterpenes as compounds in their defensive secretions (Looms, 1976). Verbenol and verbenone play a role as sex pheromones in the bark beetle (Leufven et al., 1988). The yeast, *Kluyveromyces lactis* produces citronellol, linalool, and geraniol (Kempler, 1983).

2. Classification

The structure of most terpenes follows the “isoprene rule” that was first propounded by Wallach in 1887 (Erman, 1985). This rule states that most terpenoids can be constructed by a “head-to-tail” joining of isoprene units. This principle was a major advance in terpene chemistry as it provided the first unified concept for a common structural relationship among terpene natural products (Croteau, 1980). This rule leads to the classification of the terpenes by the number of isoprene units.

Number of isoprenes		
Monoterpenes	C ₁₀	2
Sesquiterpenes	C ₁₅	3
Diterpenes	C ₂₀	4
Sesterterpenes	C ₂₅	5
Triterpenes	C ₃₀	6
Carotenoids	C ₄₀	8
Rubber	(C ₅) _n	n (Hanson, 1972)

As the structures of increasing numbers of terpenes were elucidated, it became apparent that not all terpenes directly conformed to the “isoprene rule”. Some terpenes were not exact multiples of five. Some showed a “head-to-head” arrangement of isoprene units rather than the regular “head-to-tail” pattern, while others did not fit either pattern. This led Ruzicka in 1953 to formulate the “biogenetic isoprene rule”. The rule states that higher terpenes are formed by head-to-head coupling of farnesyl or geranyl-geranyl derivatives. Ruzicka also proposed the theory that all terpenes are derived from geraniol, farnesol, geranylgeraniol or similar isoprenoids either directly or by cyclization, rearrangement or dimerization (Erman, 1985). This hypothesis ignored the precise character of the biological precursors and assumed only that they are isoprenoid in structure and is largely based on mechanistic considerations. According to this, the origin of monoterpenes is from one of four kinds of skeleton structures (Figure 1) (Croteau, 1980).

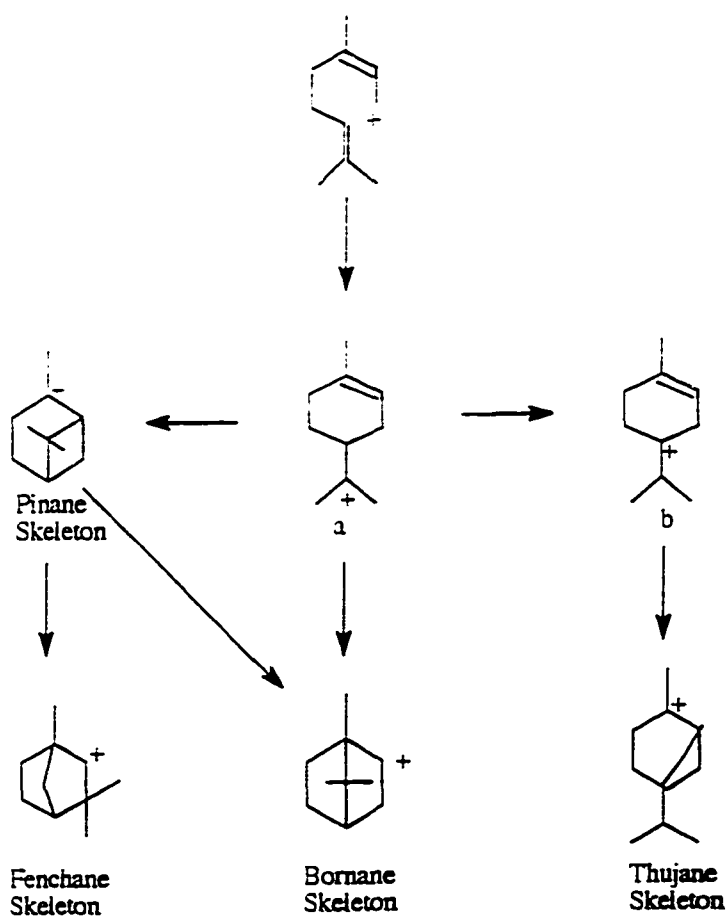


Fig. 1. Ionic scheme for the formation of monoterpenes via the α -terpinyl cation (a) and terpinen-4-yl cation (b).

3. Industrial uses of the terpenes

The “essential oils” and specific terpenes isolated from these oils, have been used in pharmaceutical, flavor, and perfume preparations since the Middle Ages. With the development of the modern chemical industry during the 19th and 20th centuries, the major natural terpenes have served as intermediates in the preparation of a wide variety of compounds. They have been used in the plastics, paint, adhesives, food, pesticide, agriculture, soap, detergent, meat-packing, paper, perfume, toiletry, rubber, textile, tobacco, as well as numerous diversified industries (Erman, 1985). The largest current use of the “essential oils” and their terpene isolates is in the compounding of flavors and perfumes for the cosmetic, soap, and food industries. The range of odor and flavor properties of the terpenes listed in Table 1 provide some indication of why terpenes are essential to the formulation of specific odor and flavor formulations.

Although each “essential oil” is a composite of hundreds of individual components, specific terpenes are generally responsible for the unique organoleptic property of each “essential oil”. Thus, the prominent odor of camphor oil is due to the powerful odor of the monoterpene camphor; while the basic scent of rose oil is due to the fragrance of the monoterpenes, geraniol and citronellol.

4. Monoterpenes

Monoterpenes are defined as molecules containing ten carbon atoms, derived from the dimerization of two molecules of isoprene, usually in a head to tail fashion, sometimes followed by oxidation, reduction, rearrangement or other chemical reactions (Erickson, 1976). Monoterpenes are classified according to their structures (Table 2).

Table 1.
Organoleptic properties of terpenes

Terpene	Odor or flavor
d-limonene	orange
l-limonene	lemon
d-carvone	caraway
l-carvone	spearmint
l-menthol	peppermint
α -pinene	pine
camphor	camphor
irones	violet
ionone	violet
santalols	sandalwood
linalool	lily of the valley
geraniol	rose
citronellol	rose
cedrol	cedarwood
citral	lemon
nootkatone	grapefruit
α -terpineol	lilac

(Erman, 1985).

Table 2.
Structural categories for monoterpenes

	Acyclic	Monocyclic	Bicyclic
Hydrocarbons	Ocimene	Limonene	Sabinene
	Myrcene	Phellandrenes	Thujene
		Terpinenes	Pinenes
		Terpinolene	Carenes
		p-Cymen	
Alcohol	Nerol	Carveol	Sabinol
	Geraniol	Menthol	Thujyl alcohol
	Citronellol	Piperitol	Myrtenol
	Linalool	Isopulegol	Fenchyl alcohol
		Terpineol	Borneol
		Carvacrol	
		Thymol	
		Pulegol	
Aldehydes	Citral	Perillaldehyde	
	Citronellal	Phellandral	
Ketones		Carvone	Unbellulone
		Dihydrocarvone	Thujone
		Piperitone	Fenchone
		Carvomenthone	Camphor
		Carvotanacetone	
		Pulegone	
		Isopulegone	
Esters	Linalyl acetate	Terpinyl acetate	Bornyl acetate

Source: Adapted from Erickson (1976).

Monoterpenes constitute the lower boiling fraction of the “essential oils”. Because of their volatility, the monoterpenes and the sesquiterpenes contribute materially to the odor and flavor of essential oils (Croteau, 1980). Monoterpenes are categorized into three groups: acyclic monoterpenes; monocyclic monoterpenes, and bicyclic monoterpenes (Whittaker, 1972). The acyclic monoterpenes are derivatives of the saturated hydrocarbon, 2,6-dimethyloctane. Most of the naturally occurring acyclic monoterpenes have pleasant odors, so they have been synthesized on the large scale for use in the perfume industry (Fordham, 1968). Monocyclic monoterpenes may be regarded conformationally as substituted cyclohexanes in which the isopropyl substituent, being bulkier than the methyl, assumes the equatorial position. Fourteen dienes having the p-menthane skeleton can theoretically exist, and all have been synthesized. Only six definitely occur in nature, these being limonene, terpineolene, α -terpiene, γ -terpiene, α -phellandrene and β -phellandrene. Bicyclic monoterpenes, as the name indicates, contain two rings in their structure (Whittaker, 1972). He further divided bicyclic monoterpenes into five subgroups: carane series; thujane series; pinane series; camphane series; and fenchane series.

5. Limonene

Limonene ($C_{10}H_{16}$) is the most widespread terpene in nature. It has been found in more than 300 essential oils in amounts ranging from a high of 90%-95% (lemon, orange, mandarin) to as low as 1% (palmarosa). The most common form is d-limonene, followed by the racemic mixture, and then l-limonene (Arctander, 1969). Limonene is a liquid with lemon-like odor. It is a highly reactive compound. Oxidation reactions often

yield more than one product. The limonenes are used as fragrance materials in household products and as components of artificial “essential oils”. By far, the largest quantities are employed as raw materials in the manufacture of terpene alcohols and ketones (Bauer and Garbe, 1985).

The primary sources of d-(+)-limonene are the process streams from citrus fruit. It is decanted from evaporator condensate streams during concentration of citrus peel press liquor to molasses, steam distilled from emulsions during cold-pressed peel oil processing or recovered as a product of essential oil concentration. (+)-Limonene is utilized as a raw material for chemical syntheses of terpene resin adhesives and flavor chemicals. Other applications include uses as a solvent in waterless hand cleaners, pet shampoo and degreasing agents (Matthews and Braddock, 1987). The annual world availability of d-(+)-limonene amounts to about 50 million kg and is primarily dependent on the amount of oranges processed in the major citrus growing regions of Florida and Brazil (Braddock and Cadwallader, 1995). Limonene from citrus is relatively inexpensive, the price is approximately 60% lower than that of α -terpineol (Anon, 1990).

6. α -Tepineol

Commercially α -Tepineol ($C_{10}H_{18}O$) is probably the most important monocyclic monoterpene alcohol. It is a colorless, crystalline solid, smelling of lilac. The most commonly available terpeneol is a liquid mixture of isomers, mainly containing α -terpineol with small amounts of l-terpinen-4-ol. α -Tepineol has been found in derivatives of more than 150 leaves, herbs, and flowers. The d-form is commonly found in star anise, marjoram, clary sage, and neroli. The l-form is found in lavandin, cajeput,

lime, lemon, cinamon, and the distillate from *Pineceae*. The racemic form is found in cajone linalool and cajeput (Arctander, 1969).

Although α -terpineol occurs in many “essential oils”, only small quantities are actually produced. A common commercial process is hydration, by aqueous mineral acid, of pinene or turpentine oil to the easily-crystallized cis-terpin hydrate (mp 117°C), followed by partial dehydration to α -terpineol (Bauer and Garbe, 1985).

α -Terpineol is an important flavor and fragrance chemical. Major uses include various flavor compositions, such as berry, lemon, lime, nutmeg, orange, ginger, anise, peach, etc. It’s annual consumption for flavor purposes has been estimated at over 13,000 kg, which places it among the top 30 commonly used flavors (Welsh et al, 1989).

II. Microbial bioconversion of monoterpenes

1. Definition of biconversion or biotransformation

Biotechnological approaches for the synthesis of flavors and fragrances are divisible into two classes according to the production method: microbiological or enzymatic. Microbiological methods can be subdivided into biosynthesis or biotransformation. Biosynthesis refers to the production of chemical compounds by metabolizing cells (fermentation and secondary metabolism), whereas biotransformation is defined as using microbial cells to perform enzymatic modifications or interconversion of chemical structures (Welsh, et al., 1989).

For many years, plants were the sole source of flavor compounds and most flavors were isolated from different “essential oils”. However, sensorially active compounds are

often present only in minor quantities or in a bound form, making their isolation difficult. Due to increasing requirements of food industries for natural flavor materials, and limited availability from natural sources, flavor production by microorganisms has been raised from a laboratory oddity to an industrial possibility (Kempler, 1983, Drawert, 1988, Manley, 1987, Gatfield, 1988, Schreier, 1989, Janssens, et al., 1992).

Terpenes are favored substrates for bioconversions studies because a significant number of the fragrances and flavors are monoterpenoids, sesquiterpenoids or analogous structures. The specific properties of these compounds depend on the absolute configuration of their structures. Therefore, the synthesis of these substances requires reactions with high regio- and enantio-selectivities and may require methods for the introduction of one or more chiral centers.

2. Methods for bioconversion

Bioconversion techniques can be categorized as those using whole cells or isolated enzymes (Welsh et al., 1989). A summary of the advantages and disadvantages of bioconversions of whole cells versus isolated enzymes is given in Table 3.

Jones, et al. (1976) sketched the steps for carrying out a bioconversion using growing cells as: 1) selection of the desired organism; 2) growth of the culture in a medium supporting maximal cell formation; 3) addition of the substrate to the growing cell suspension; and 4) continuation of the incubation of the cell mass and substrate until the maximum conversion has been achieved. The advantages of this method are the relative ease of operation and high conversions. The disadvantages of this method are screening for a desirable culture, contamination, and a relatively high equipment

Table 3.
Advantages and disadvantages of using isolated enzymes and whole cell systems

Types	Forms	Advantages	Disadvantages
Enzymes	Isolated enzymes in general	Simple apparatus, simple work-up, better productivity due to higher concentration tolerance	Cofactor recycling necessary
	Dissolved water	high enzyme activity	Side reaction possible, lipophilic substrates insoluble, work-up requires extraction
	Suspended in organic solvents	Easy to perform, easy work-up, lipophilic substrates soluble, enzyme recovery easy	Low activities
	Immobilized	Enzyme recovery easy	Loss of activity during immobilization
Whole cells	Using whole cells in general	Expensive equipment, tedious work-up due to large volumes, low productivity due to lower concentration tolerance, low tolerance of organic solvents, side reactions likely due to metabolism	No cofactor recycling necessary
	Growing cells	Higher activities	Large biomass, more by-products
	Resting cells	Work-up easier, fewer products	Lower activities
	Immobilized cells	Cell reuse possible	Low activities

(From Faber 1992).

requirement. Much research has been done on using growing cells for bioconversions. Abraham (1994) reported that 40 strains of bacteria and 60 fungi screened for bioconversion of three substrates using the above methods.

Resting cells are live, non-dividing cells that retain many of their enzyme activities (Rosazza, 1982). The major advantages of using resting cell suspensions are: 1) separation between growing and transformation phases; 2) reduced substrate toxicity; 3) avoidance of side reactions (Jones et al., 1976). Finkelstein and Ball (1992) summarized the procedures of fungal bioconversions by using resting cells and growing cells in Figure 2. Yoo (1993) used resting cells of a *Pseudomonas* strain (P#8#1) isolated from soils surrounding pine trees for bioconversion of (\pm)- α -pinene. The five major products limonene, p-cymene, α -terpineolene, α -terpineol, and endo-borneol were identified.

Immobilized fungi have been used to transform steroids. Ceen et al. (1987) used immobilized *Aspergillus ochraceus* to study bioconversion of 11 α -hydroxylation in progesterone. Schlosser and Schmauder (1991) reported a bioconversion of 13-ethyl-gon-4-ene-3,17-dione by immobilized *Penicillium raistrickii*.

3. Bacteria and fungal bioconversion of monoterpenes

(1). Limonene

The first reports on a bacterial biotransformation of limonene were by Dhavakar and Bhattacharyya (1966a). Limonene 1% (v/v) was added during the fermentation of a soil bacteria. The following products were found; dihydrocarvone, carvone, carveol, 8-p-menthene-1,2-diol, 8-p-menthen-1-ol-2-one, 8-p-menthene-1,2-trans-diol, and 1-p-menthene-6,9-diol; perillic acid, β -isopropenyl pimelic acid, 2-hydroxy-8-p-menthen-7-

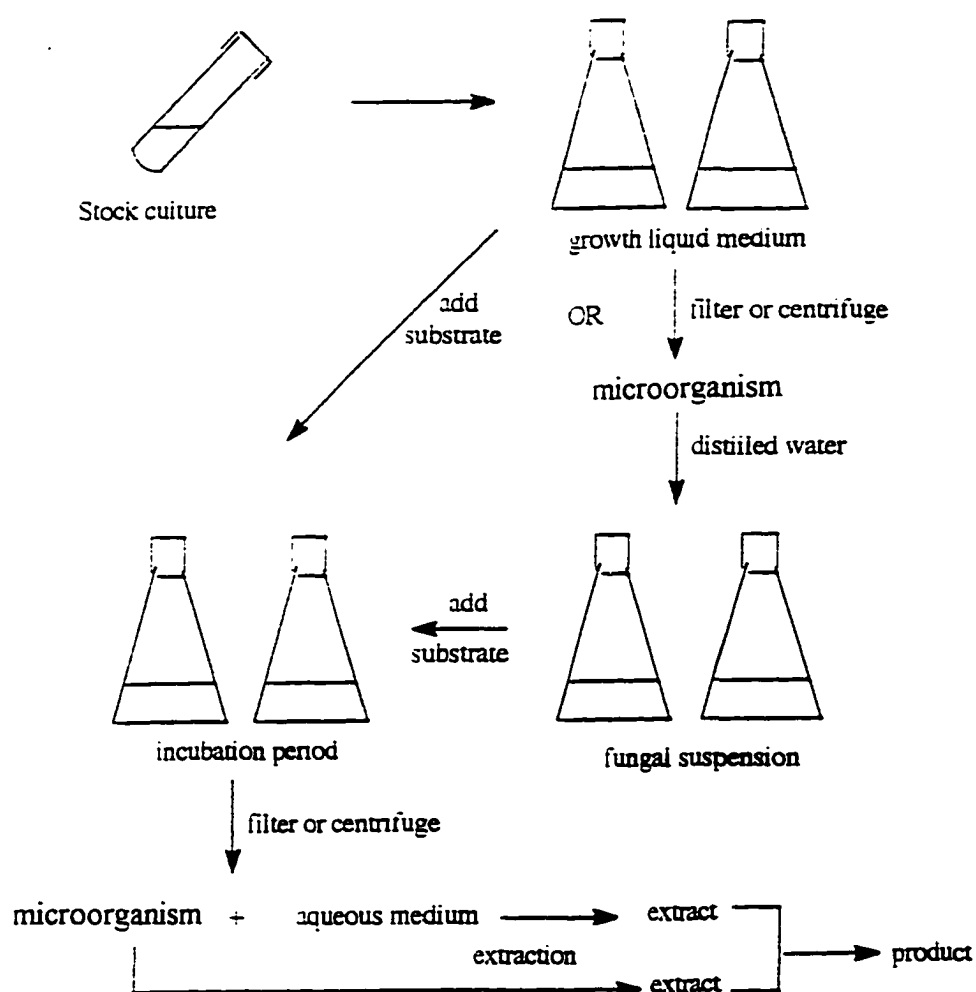


Fig. 2. Standard procedures for bioconversion with fungi.

oic acid and 6,9-dihydroxy-1-p-methene-7-oic acid. In a subsequent study, the authors (1966b) proposed three pathways for this biotransformation of limonene (Figure 3). Pathway 1 produces (+)-cis-carveol, (+)-cis-carvone, and 1-p-menthene-6,9-diol. Pathway 2 produces (+)-dihydrocarvone via an epoxide intermediate, and 8-p-menthene-1-ol-2-one. Pathway 3 produces 2-hydroxy-8-p-menthene-7-oic, isopropenyl-pimelic acid, and 4,9-dihydroxy-1-p-menthene-7-oic acid.

A different bioconversion pathway for limonene for a *Cladosporium* species was reported by Kraidman et al. (1969). They found that the double bond of limonene was monohydroxylated to produce α -terpineol. The product, D- α -terpineol, was obtained at a concentration about 1 g/L when the microorganism was grown on limonene as the C-source. This microbial transformation was reported to have an advantage over chemical synthesis because there was no β -terpineol as a side product.

Another microbial transformation of limonene, by a *Cladosporium* sp., was reported by Mukherjee et al. (1973). The yield of the major product, limonene-1,2-*trans*-diol, was about 1.5 g/L with less than 0.2 g/L of a minor product corresponding to limonene-1,2-*cis*-diol. According to the authors, the potential of this bioconversion was that the product, limonene-1,2-diols, can be used as a precursor to carvone, an important spearmint flavor

Based on these reports, the pathways of limonene degradations by microorganisms were updated (Figure 4). Many other microbial strains have been found to degrade limonene. *Chaetomium cochlioids* DSM 1909 or *Chaetomium globosum* DSM 62109 degraded (+)-R-limonene producing trans-carveol instead of the normal (+)-cis-carveol as

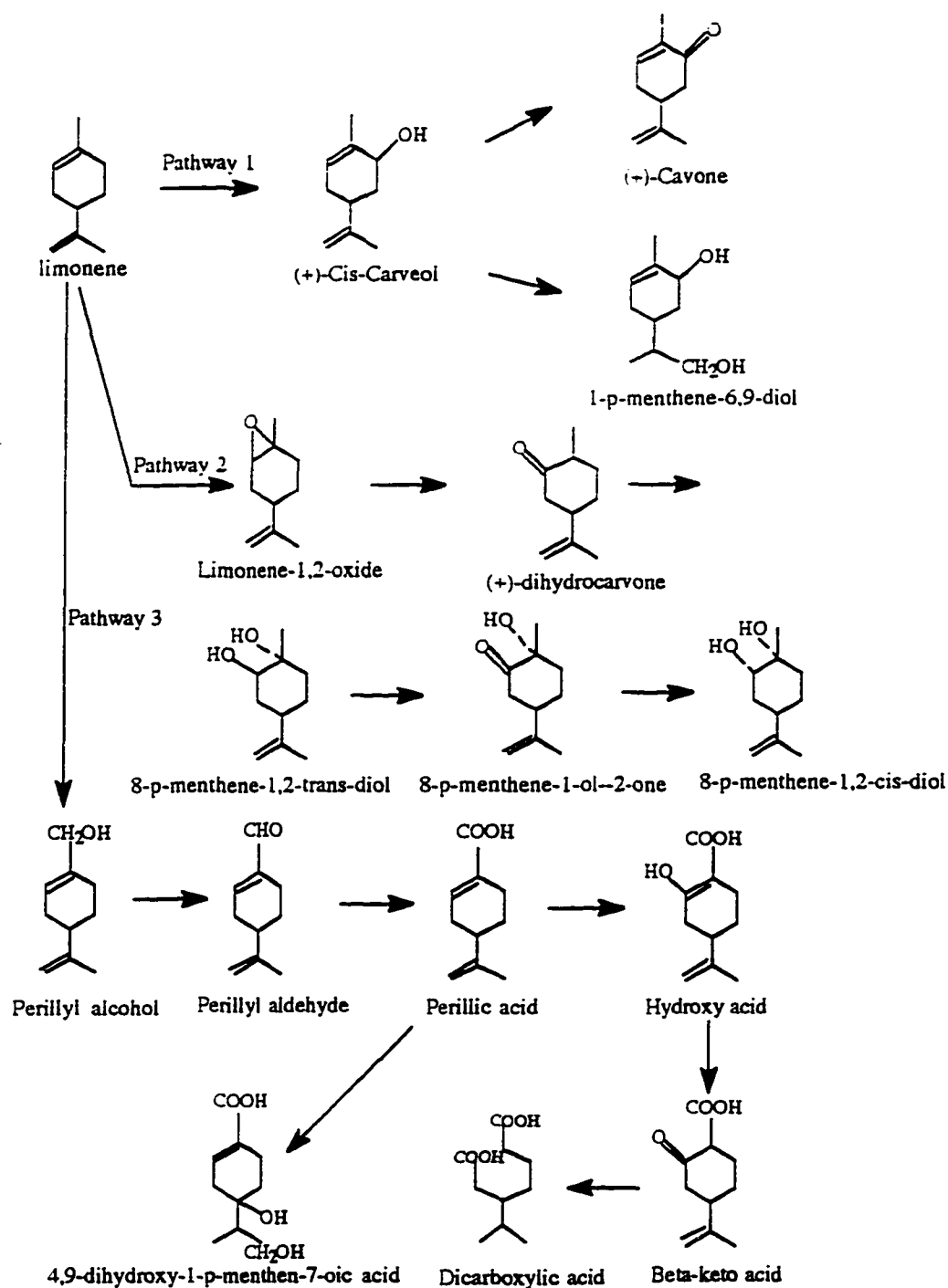


Fig. 3. Pathways of degradation of limonene (after Dhavalihar, et al., 1966).

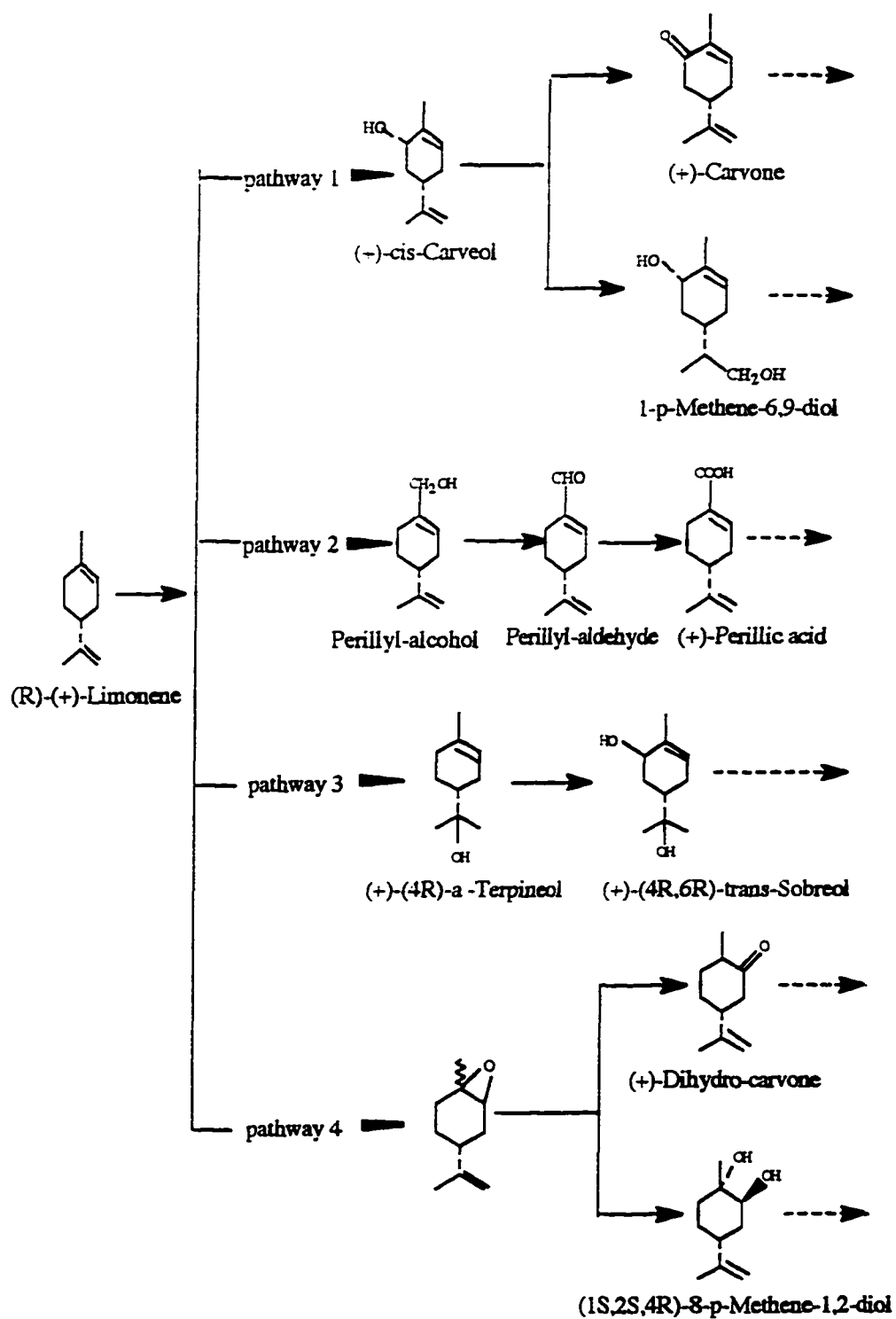


Fig. 4. Pathways of microbial degradation of (R)-(+)-limonene.

the primary metabolite (Kieslich, et al., 1986). These fungi also transformed (-)-S-limonene into a mixture of 6S- and 6R-carveols. *Penicillium digitatum* (DSM 62840) converted a racemic limonene into (+)- α -terpineol (Stumpf et al., 1983). A similar conversion of limonene into 8-p-menthene-1,2-diol and tetramethyl-limonene into 8,9-epoxy-3,3,5,5-tetramethyl-1-menthenol-6 was conducted with *Gibberella cyanea* (Abraham et al., 1986). The epoxidation at 8,9-position was not completely stereoselective because a 2:1 mixture of these epoxides were found.

4R-(+)- α -Terpineol and 4R-(+)-perillic acid were major products of the conversion of limonene by a strain of *Pseudomonas gladioli* (Cadwallader et al., 1989). The strain was isolated from pine bark and sap by enrichment culture. The maximum concentration of α -terpineol and perillic acid after 4 days fermentation was 702 ppm and 1861 ppm, respectively. Perillic acid production was thought to be an intermediate of energy yielding pathway for limonene degradation. In a subsequent study, the enzyme α -terpineol dehydratase (α -TD) was isolated by cell disruption and detergent extraction. Characteristics of this enzyme were reported by Cadwallader and Braddock (1992).

Bioconversion of limonene by the fungus of *Aspergillus cellulosa* M-77 was investigated by Noma, et al. (1992). Their results showed that (+)-limonene was transformed mainly to (+)-isopiperitenone, (+)-limonene-1,2-trans-diol, (+)-cis-carveol and (+)-perillyl alcohol, whereas (-)-limonene was transformed into (-)-perillyl alcohol, (-)-limonene-1,2-trans-diol and (+)-neodihydrocarveol as the major products. Thus, this bioconversion via the fungus is not stereoselective but stereospecific.

(2). Pinene

Studies concerning the microbial biotransformations of α -pinene started in the early 1960's (Bhattachacharyya et al, 1960). A strain of *Aspergillus niger* was isolated from the infected bark of agarwood and grown on a medium enriched with 0.5% of α -pinene. The α -pinene substrate employed in the experiment consisted of a mixture of 70% of (+)- and 30% of (-)- α -pinene. Product yields, on the basis of pinene utilized, were 20-50% verbenol, 2-3% verbenone, and 2-3% sobrerol. It was suggested that the formation of cis-verbenol resulted either from a free radical attack at the allylic position or by removal of hydride ion from the same position through mediation of an electrophilic species. In case of (+)-trans-sobrerol, the attack on the 1,2 double bond by an electron deficient oxygen was postulated.

In a subsequent study (Prema and Bhattacharyya, 1962) with the same strain, it was found that α -pinene had an inhibitory concentration. The optimal period of incubation, leading to maximal yields of products was 6 to 8 hours. The transformation of α -pinene by this strain was very sensitive to changes in temperature, where a temperature of 27-28°C resulted in maximum yield of oxygenated products, with transformation being negligible above 30°C. Three major metabolites were isolated; a ketone, 2-3% (+)-verbenone; an alcohol, 20-25% (+)-cis-verbenol; and a crystalline diol, 2-3% (+)-trans-sobrerol. It was concluded that verbenone and verbenol were the true transformation products of this mold. In addition, it was found that all the metabolic products were enantiomerically pure and probably were derived from (+)- α -pinene. However, in light of absence of change in specific rotation of α -pinene before and after fermentation, they

postulated that (-)- α -pinene was metabolized by a different pathway. They also suggested that the oxygenated products were further broken down to carbon dioxide and water. These studies established that terpenes when used as substrates were transformed into hydroxylated, reduced, oxidized, or degraded products by microorganisms (Bhattacharyya and Ganapathy, 1965).

Many *Pseudomonas* strains have been found that are able to convert α - and β -pinene. Microbiological transformations of α - and β -pinene by a soil *Pseudomonad* (PL-strain) were studied by Shulka et al. (1968). This organism utilized α -pinene, β -pinene, limonene 1-p-menthene, or p-cymen as sole carbon source. Fermentation of (+)- α -pinene resulted in the formation of a large number of acidic and neutral metabolites, including (+)-borneol, myrtenol, (+)-oleuropeic acid, (-)- β -isopropyl pimelic acid, myrtenic acid, phellandric acid, perillic acid, 4-hydroxydihydrophellandric acid, and 4,9-dihydroxy-1-p-methen-7-oic acid.

The pathways of degradation of α - and β -pinene were evaluated for this bacterium (PL-strain). Growth and adaptation studies suggested that there were at least six different pathways for α -pinene metabolism. One pathway involved the progressive oxidation of the 7-methyl group of pinene to yield mythenol, mythenal, and mytenic acid. The other pathways were initiated by an attack of a proton on the double bond of α -pinene, leading to a common pinyl cation and its rearranged products, p-menthene-8 and 4-cation. It was concluded that the reduction of the 4-cation to yield 1-p-menthene was the rate-limiting step in the sequence and the lack of sufficient amounts of a hydride donor, such as

reduced NADP, under anaerobic conditions inhibited the protonation reaction (Shukla and Bhattacharyya, 1968).

In a separate study by Joglekar et al. (1969), two strains of soil *Pseudomonas*, RDP-Red and RDP-White, were isolated from sewage. Transformation of α -pinene by RDP-Red strain resulted in an acidic fraction composed mainly of citronellic acid (8%) and a neutral fraction composed of unidentified products. Transformation of α -pinene by RDP-White strain resulted in an acid fraction containing of citronellic acid (2%) and neutral fraction also composed of unidentified products.

Gibbon et al. (1971) reported that the degradation of α -pinene by *Pseudomonas* PX1 was different from that exhibited by the PL strain studied by Shukla and Bhattacharyya (1968). They proposed a pathway for α -pinene degradation by *Pseudomonas* PX1 on the basis of the acidic bioconversion products, since only one of the neutral products, (+)-trans-carveol, was identified. In a further study, two *Pseudomonas* sp. (PX1 and PIN18) were isolated from soil with α -pinene as the sole carbon source, *Pseudomonas* PX1 produced two neutral products, *cis*-thujone and *trans*-carveol from α -pinene and *Pseudomonas* PIN18 produced terpinolene, limonene and borneol (Gibbon et al., 1972). It was found that (+)-pinene was utilized in preference to the (-)-pinene producing predominantly the (+)-isomer of limonene in this study.

Two novel metabolites produced in α -pinene metabolism by a similar organism, *P. putida* PIN 11, was reported by Tudroszen et al. (1977). They were 3-isopropylbut-3-enoic acid and (Z)-2-methyl-5-isopropylhexa-2,5-dienoic acid. The majority of *PIN* mutants, *P. putida* PIN 11, were found to accumulate the same products as the wild-type

organism, but in greater overall yield and different relative amounts. These mutants were obtained by treating the parent *P. putida* PIN 11 with N-methyl-N'-nitro-N-nitrosoguanidine.

Utilization of β -pinene by *Pseudomonas pseudomallai* was studied by Dhavlikar et al. (1974). Metabolic products were found to be medium dependent. Isoborneol, camphor, borneol, α -terpineol, and β -isopropyl pimelic acid were formed when Seubert's mineral medium was used. The yields of neutral and acidic products were 2% to 2.5% and 1%, respectively. However, *trans*-pinocarveol, mytenol, α -fenchol, α -terpineol, and mytenic acid were produced on Czapeck-Dox medium. The yield of the neutral and acidic transformation products was correspondingly changed to 2.5% and 0.5%, respectively.

Pseudomonas maltophilia was shown to transform α -pinene into limonene, borneol, camphor, perillic acid, and 2-(4-methyl-3-cyclohexenylidene) propionic acid (Narushima et al., 1982). The quantity of the neutral residues extracted from a reaction of mixture of α -pinene (10 ml) and resting cells after 48 hr grown at 30°C was 2.1 g and consisted of limonene, camphor, and borneol. The quantity of the acid residues extracted from the culture broth (15 L) was 3.2 g and consisted of methyl phellandrate, methyl perillate, and propionic acids. Metabolic oxidation of α -pinene by *Acetobacter methanolicus* has been shown to transform (+)- α -pinene into mainly (+)-*trans*-verbenol (14%), verbenone, *trans*-pinocarveol, and *trans*-sobrerol (Weber et al., 1988).

Transformation of α -pinene and β -pinene by *Arillariella mellea* (honey fungus), a parasite of woodlands was studied by Draczynka, et al. (1985). The substrates were α -pinene and β -pinene, which are major turpentine components. The study suggested that

the biotransformation of opposite enantiomeric substrates resulted in different products. The proposed transformation of (+)- α -pinene yielded a mixture of ten compounds. The structural selectivity of the enzymes of *A. mellea* could be demonstrated by comparing the results obtained by bioconversion of (-)- β -pinene. The main products were trans-pinocarveol, isopinocampone, pinocarvone, 7-hydroxy- α -terpineol, and 1-hydroxy-pinocarveol. Through study of the enantioselectivity of the metabolism of some monoterpenic components, the same fungus was shown to oxidize (\pm)- α -pinene via α -terpineol to (4S)-trans-sobrerol faster than to the (4R)-isomer (Draczynska, et al., 1989).

Griffiths et al. (1987a) isolated a *Nocardia* sp. (strain P18.3) from a group of over 20 gram-positive bacteria by selective culture with (\pm)- α -pinene as the sole carbon source. Metabolites, produced during growth by this strain from (\pm)- α -pinene, were tentatively identified by GC/MS as isoborneol, 1,8-cineol, menth-1-en-6-one, menthone, pinocampone, iso-pinocampone, thujone, verbenol, and verbenone. α -Pinene oxide was found to be an intermediate in the degradation of α -pinene by both this strain and some other organisms. This epoxide was cleaved by a lyase which catalyzes a concerted reaction in which both rings of the bicyclic structure are cleaved with the formation of cis-2-methyl-5-isopropylhexa-2,5-dienal (Griffiths et al., 1987b).

(3). Other monoterpenes

Biotransformation of citronellal and citral by *Pseudomonas aeruginosa* strain RDC-1 was reported by Joglekar et al. (1968). Biotransformation of citronellal through the organism produced citronellic acid, citronellol, dihydrocitronell, menthol, and 2,6-dimethyl octen-6,8-diol after 4 days fermentation with yield of 65%, 0.6%, 0.6%, 0.75%

and 1.7%, respectively. The products from citral biotransformation by the bacterium were geranic acid, 6-methyl-hept-6-enloic acid, dimethyl acrylic acid, 2-6-dimethyl- Δ^2 -octen-7-one-8-ol, after 2 days fermentation, with yields of 62%, 0.5%, 1%, and 0.75%, respectively. The cyclization of citronellal with subsequent hydrogenation to menthol by *Penicillium digitatum* was patented by a research group in Czechoslovakia early in 1952 (Kieslich et al., 1985).

Microbial oxidation of β -ionone by *Aspergillus niger* was patented by Krasnobajew and coworkers (Krasnobajew, 1978) at Givaudan Company. This process has potential for use in flavoring tobacco. In another study, they (Krasnobajew, 1982) reported the bioconversion of β -ionone via hydroxylations and oxidations into a mixture of metabolites useful as tobacco flavoring using the fungus *Lasiodiaplodia theobromae* (ATCC 28570). Up to 10g β -ionone per liter medium can be converted with a yield of 90%.

A *Nocardia* sp. produced cumic acid from p-cymene was reported by Davis and Raymond (1961). The yield of cumic acid was 10% after 5 days fermentation. From a broth culture, a crystalline precipitate 0.5 g from 5 g of substrate formed when the concentrated broth culture was made acidic to pH 2 to 3 with HCl.

Asakawa et al. (1991) reported biotransformation of four different monoterpenoids by an *Aspergillus* species. *A. niger* converted (-)-menthol to 1-, 2-, 6-, 7-, and 9-hydroxymenthols and the mosquito repellent-active 8-hydroxymenthol. While (+)-menthol was biotransformed to give 7-hydroxymenthol. *Aspergillus cellulosa* biotransformed (-)-menthol specifically to 4-hydroxymenthol. Terpinolene and (-)-

carvotanacetone were converted by *A. niger* to two α,β -unsaturated ketones, a fenchane-type compound and distereoisomeric p-menthane-2,9-diols and 8-hydroxycarvomenthol, respectively.

III. Microbial systems for hydroxylation reactions

1. Microbiological hydroxylation

Microbial hydroxylation plays an important role in degradation of many organic compounds. This reaction has been studied for the microbial utilization and transformation of hydrocarbons from petroleum industry, for degradation of wastes or production of single cell proteins (SCP); microbial hydroxylation of steroids for pharmaceutical purposes; or microbial hydroxylation of terpenoids for flavor and fragrance industrial research. Recently, there have been numerous reports on the potential of microbial hydroxylation reactions for the large scale preparation of regio- and stereoselective hydroxylated metabolites of natural or synthetic complex molecules of pharmacological interest and the preparation of functionalized asymmetric synthons of high optical purity. Biohydroxylation reactions represent a powerful technique for the introduction of functional groups into already elaborated molecules, with the benefit of the regio- and stereoselectivity of enzymatic reactions (Aszerad, 1993).

2. Microbial hydroxylation of aliphatic and aromatic compounds

In 1970, Cardini and Jurtshuk reported that cell-free extracts from sonically disrupted *Corynebacterium sp.* strain 7E1C (ATCC 19067) oxidize n-octane to 1-octanol and octanic acid in the presence of NADH and O₂. They showed that molecular oxygen

was incorporated into the substrate during hydroxylation. Two protein fractions of the hydroxylation system were isolated. One fraction contained cytochrome P-450 and the other contained a flavoprotein. Colby et al. (1977) reported that *Methylococcus capsulatus* (Bath) oxygenates n-alkanes, n-alkenes, ethers, and alicyclic, aromatic and heterocyclic compounds. In their study, C1-C8 n-alkanes were hydroxylated, yielding mixtures of the corresponding 1- and 2-alcohols, but no 3-or 4-alcohols are formed.

Microbial hydroxylations are now used in pharmaceutical manufacture. An important application is steroids modification (Schmauder, et al., 1991). It has long been known that fungus *Rhizopus sp.* performs hydroxylations of steroids. Holland and Auret (1975) reported the Δ^4 -3-ketosteroid analog (\pm)-4a-methyl-4,4a,5,6,7,8-hexahydro-2(3H)-naphthalenone is hydroxylated at both C-8 α and C-8 β by *Rhizopus arrhizus* (ATCC 11145). A mechanism was proposed for both microbial and chemical hydroxylation. Maddox et al. (1981) first used immobilized *Rhizopus nigricans* cells to produce an 11 α -hydroxylation of progesterone. Vidyarthi and Nagar (1994) immobilized the same fungus by using polyacrylamide, agar or chitosan matrices to produce 11 α -hydroxylate progesterone. In 1984, Fukui et al. using another strain, *Rhizopus stolonifer*, produced the same 11 α -hydroxylate progesterone. In 1991, Ouazzani et al. used *Rhizopus arrhizus* to hydroxylate octalin derivatives at allylic positions with high yields.

Every site in a steroid molecule is accessible for hydroxylation, the 11 α -, 11 β - and 16 α -hydroxylations are now exclusively conducted by microbial transformations (Mahato and Majumdar, 1993). Much research has been conducted on microbial hydroxylation of cholesterol for production of precursors in steroid manufacturing, such

as 4-androsten-3,17-dione (AD) and /or 1,4-androstadien-3,17-dione (ADD) (Smith, et al., 1993). Other hydroxylations which have potential for industrial exploitation are 7 α -, 9 α -, and 14 α -hydroxylations. Microbial 9 α -hydroxylation of androst-4-ene-3,17-dione (AD) has opened up a means of preparing $\Delta^{9,11}$ -intermediates which are subsequently transformed chemically to 9 α -fluorocorticoids, by-passing the traditional microbial 11-hydroxylation. *Nocardia rhodocrous* cells transform AD to ADD (Omata et al, 1979). Immobilized *Rhodococcus equi* DSM 89-133 cells converted cholesterol into AD and ADD (Ahmad and Johri, 1992). Tween 80 was found to improve cholesterol oxidation by *Mycobacterium* sp. into 4-cholesten-3-one (cholestenone), 4-androsten-3,17-dione (AD), 1,4-androstadien-3,17-dione (ADD), testosterone, and 1-dehydrotestosterone (DHT) (Smith et al., (1993).

The 15 α -hydroxylation of 13-ethyl-gon-4-ene-3,17-dione was first discovered by Petzoldt and Wiechert (1976) using fungal mycelia of *Penicillium raistrickii*. The 15 α -hydroxylation product is a crucial intermediate for the production of pharmaceutical useful steroids, e.g. gestodene. Schlosser and Schmauder (1991) reported production of the product by this fungus immobilized onto calcium alginate gel beads.

Fungal hydroxylation of aromatic compounds have been extensively exploited (Holland et al., 1985, 1986, 1987, 1988a, 1988b, 1990, 1993). Fungal hydroxylation of ethyl benzene and derivatives by *Mortierella isabellina* has been shown (Holland et al., 1985). This fungus converts ethyl benzene and a number of para-substituted derivatives to the corresponding optically active 1-phenylethanols with enantiomeric excesses between 5 and 40%. In another study, *Mortierlla isabellina*, *Cunninghamella echinulata* var.

elegans and *Helminthosporium* sp. were all found capable of performing biotransformations of ethylbenzene and a number of para-substituted derivatives (Holland et al., 1986). The highest enantiomeric excesses during benzylic hydroxylation were obtained with *Helminthosporium* and were attributable, at least in part, to further stereoselective oxidation of the alcohol. The same research group (1988) reported side chain hydroxylation of aromatic compounds by fungus *Mortierella isabellina*. In all cases, the rate of product accumulation was uniform over a period of at least 4 days.

3. Microbial hydroxylation of terpenoids

Many natural terpenoid compounds are common, inexpensive, substances which are used as starting materials for organic synthesis (Azerad, 1993). Various microorganisms have been reported to hydroxylate terpenoids (Janssens et al., 1992; Mahato and Majumdar, 1993; Abraham, 1994; Larroche, et al., 1995).

An example of microbial hydroxylation for the production of flavorings is the hydroxylation of patchoulol, a sesquiterpinoid and major constituent of the patchouli oil used in perfumery. This reaction, by *Gliocladium roseum* was patented by Hoffmann-La Roche (1978) but can also be conducted with *Pithomyces* sp. (Suharta et al., 1981). Mikami et al. (1978) reported that *Aperigillus niger* preferentially hydroxylated the β -ionone substrate in the (2S)- and (4R)-position. Stumpf et al. (1982) patented a similar hydroxylation procedure using *Gongronella butleri* (CBS 15725). However, their hydroxylation product was predominantly (4R)-hydroxy- β -ionone. Krasnobajew (1982) obtained a mixture of various oxidation products in fermentations of β -ionone with *Lasiodiplodia theobromae* (ATCC 28579). This process is used by Givaudan, Inc. for

microbial oxidation of β -ionone, because these mixtures are excellent tobacco flavorings. Larroche et al. (1995) used fed-batch biotransformation techniques for production of hydroxy and oxo derivatives of β -ionone as tobacco flavorings by *Aspergillus niger* IFO 8541. The results indicated that *A. niger* IFO 8541 synthesizes 4-hydroxy- β -ionone as its main product with mass yield close to 90%.

Microbial hydroxylation may involve a biological Baeyer-Villiger pathway, an important pathway in many degradation processes (Trudgill, 1984). An example of this is the cleavage of (+)-camphor, a monoterpene compound, by *Pseudomonas putida*. Hydroxylation is the first of the sequence of reactions which is catalyzed by a soluble cytochrome P450-containing hydroxylase complex (Trudgill, 1984).

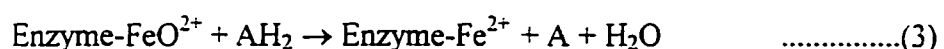
Abraham et al. (1989) reported that a strain of *Chaetomium cochlioides* produces numerous epoxides and hydroxylated derivatives from humulene, a sesquiterpene. They also indicated that hydroxylation of humulene monoepoxide proceeded more readily than hydroxylation of the hydrocarbon. Another sesquiterpene 11,13-dehydro-(-)- α -santonin (DS) has been biotransformed to several bioactive products with anti-tumor activity (Ilda, et al., 1993). Two hydroxylated santonins (11-hydroxy-(-)- α -santonin and 13-hydroxy-(-)- α -santonin) have been obtained by using *Streptomyces roseochromogens*, *Streptomyces aureofaciens*, or *Aspergillus niger* biotransformation.

4. Enzymatic systems and mechanisms related to microbial hydroxylation

It has been known for many years that enzymatic hydroxylation occurs in mammals, but only recently has the process has been studied in bacteria and fungi

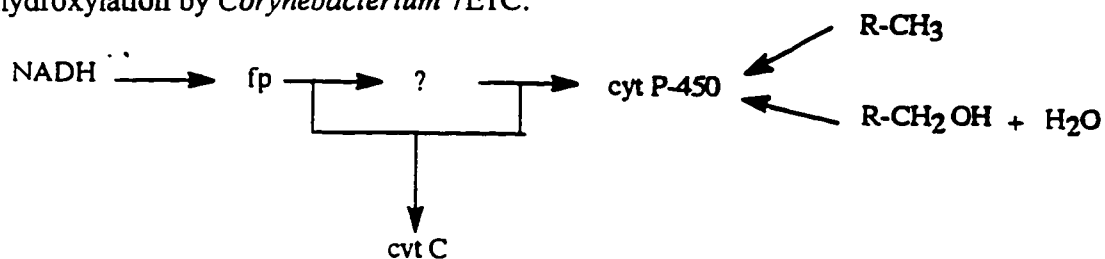
(Holland et al., 1990). The enzyme system involved in this kind of hydroxylation has been identified as a cytochrome P-450 dependent monooxygenase.

The biological hydroxylations of steroids and some aromatic compounds are carried out by 'mixed-function oxidases' which directly introduce one of the atoms of the diatomic oxygen molecule into the substrate along with the simultaneous reduction of the second atom with a suitable hydride donor such as NADH, NADPH, FMNH₂, FADH₂, ascorbic acid, tetrahydrofolate or its analogues, etc (Mason, 1957). Bhattacharyya and Ganapathy (1965) reported the following scheme after studying fungal hydroxylation reactions by *Asperigillus niger*:



The dioxygenated species, Enzyme-FeO₂²⁺, carries out the hydroxylation [Eq. (2)].

An enzyme system from *Corynebacterium* sp. Strain 7E1C was isolated by Cardini and Jurtshuk (1970) which hydroxylates n-octane. This enzyme system was separated into two protein fractions, one containing a cytochrome P-450, and the other having the spectral characteristics of a flavoprotein. Both of fractions together were required for hydroxylation activity. The authors also indicated that the cytochrome P-450 was an inducible hemoprotein. They proposed the following scheme for n-alkane hydroxylation by *Corynebacterium* 7E1C:



Peterson et al. (1968) described a soluble enzyme system from *Pseudomonas oleovorans* which converts n-octane to 1-octanol in the presence of NADH and oxygen. The system consists of three protein fractions; a rubredoxin, a reductase, and a ω -hydroxylase. Only the first two fractions were purified and characterized. An n-alkane hydroxylating system of a *Pseudomonas* sp was purified by Kusunose et al. (1968). This system also contained three fractions; a heme iron protein, a flavoprotein, and an unidentified hydroxylase. Unlike the rubredoxin-containing alkane hydroxylase of *Pseudomonas oleovorans* (May & Abbott, 1973) and the cytochrome P-450 alkane hydroxylase of a diphtheroid bacterium (Cardini and Jurtshuk, 1970), the methane mono-oxygenase of *Methylococcus capsulatus* (Bath) is not a terminal alkane hydroxylase (Colby et al. 1976, 1977). Methane mono-oxygenase oxidizes n-alkanes to mixtures of the corresponding 1- and 2-alcohols. This enzyme also hydroxylates cyclic alkanes and aromatic compounds. It requires NADPH and/or NADH as an electron donor. The methane mono-oxygenase of *M. capsulatus* (Bath) is a non-specific enzyme and many of its substrates show little or no structural resemblance to its substrate, methane. Moreover, the resistance of the methane mono-oxygenase to inhibition by CO suggests that it does not contain cytochrome P-450. It is possible that it may contain a CO-binding cytochrome c of the type involved in the analogous methane mono-oxygenase system from *Methylosinus trichosporium* (Tonge et al., 1977).

An enzyme from *Pseudomonas gladioli*, was isolated by Cadwallader et al. (1992) which hydroxylates limonene to α -terpineol. The enzyme was designated α -terpineol dehydratase (α -TD). It does not require cofactors for the hydroxylation activity. The

enzyme was partially solubilized by extraction with 10mM HEPES buffer pH 7.0 containing 2.0%(w/v) Triton X-100 and 0.5M sodium trichloroacetate. Two soluble forms existed in 1.0% Triton X-100, with apparent molecular weights of 94,500 and 206,500 daltons. Highest enzyme activity was observed at a pH of 5.5 and the enzyme was most stable at pH 8.0. This hydroxylation enzyme is different from those enzyme systems mentioned above. Braddock and Cadwallader (1995) indicated that bacteria and fungi may differ in their metabolism of terpenes. Bacteria generally metabolize (+)-limonene by progressive oxidation starting with the 7-methyl group. Generation of small amounts of neutral products which are not further metabolized may occur. Fungi attack (+)-limonene by hydration of the double bond of the isopropenyl substituent or by epoxidation -hydrolysis of the 1,2 double bond.

It is generally assumed that in alkane assimilating yeasts, the first step of alkane degradation is catalyzed by a monooxygenase system which consists of a cyt P-450 as the terminal oxidase and a NADPH-cyt P-450 reductase as the electron transfer component (Honeck et al.1982, Schunck et al., 1989, Zimmer et al., 1995). Honeck et al. (1982) purified the NADPH-cytochrome P-450 reductase from the microsomal fraction of yeast *Lodderomyces elongisporus*. One mole of enzyme contains 1 mole each of FAD and FMN and exhibits an apparent molecular weight of 79,000. Recombination of the NADPH-cytochrome P-450 reductase with highly purified cytochrome P-450 resulted in an active alkane monooxygenase system. The activity of the hexadecane hydroxylation was enhanced by the addition of non-ionic detergent. Schunck et al. (1989) indicate that the monooxygenase system from yeast *Candida maltosa*, which catalyzes the

hydroxylation of long-chain n-alkanes to fatty alcohols, is a constituent of the yeast endoplasmic reticulum. The alkane hydroxylating cytochrome P-450 from this yeast consists of 521 amino acids. There are two putative transmembrane segments in the N-terminal region and a characteristic heme-binding sequence in the C-terminal part. Zimmer et al. (1995) reconstituted individual *Candida maltosa* cytochrome P450 monooxygenase in *Saccharomyces cerevisiae*. The NADPH-cytochrome P450 reductase was from the yeast *Candida maltosa*. Three different cytochrome P450 forms were used for testing the lauric acid hydroxylation activity. The results showed that oxygen limitation (semi-anaerobic culture conditions) could enhance the P450 levels and confirmed cytochrome P450 and NADPH-cytochrome reductase are colocalized in the ER of the host organism. The optimum P450/reductase molar ratio is about 1:3 for the activity.

Fungal hydroxylation seems to be by an enzyme system similar to the yeast system. Holland et al. (1985) reported that the hydroxylation of steroids by fungi has been shown in many cases to be due to a cytochrome P-450 dependent monooxygenase reaction. The same research group (Holland et al., 1987) reported that the enzymes involved in hydroxylations of various substrates by fungi *Mortierella isabellina*, *Cunninghamella echinulata* and *Helminthosporium* species were monooxygenases. The mechanism by which the enzyme performs benzylic hydroxylation has been proposed to be either directly by hydrogen abstraction, or indirectly via a one-electron oxidation of the aromatic ring followed by proton loss in the benzylic hydroxylating process (Figure 5) (Holland et al., 1990a). The active site of the enzyme (Fe-OH) can produce the product by reaction

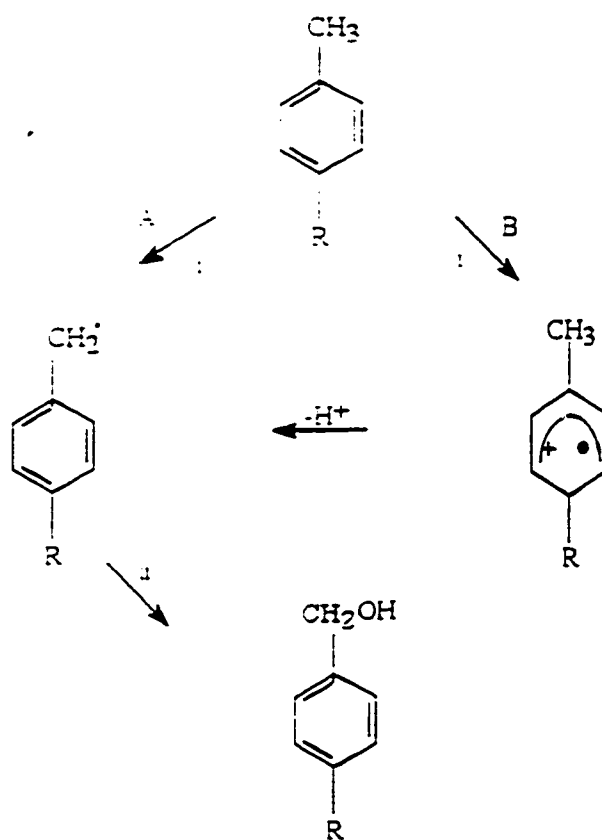


Fig. 5. Proposed routes for benzylic hydroxylation. i Fe-O•; ii Fe-OH[•]

with a benzylic radical; the latter may be produced either by abstraction of a hydrogen atom from the benzylic carbon (route A), or indirectly by loss of a proton from a radical cation generated by a one-electron oxidation of the aromatic ring (route B).

The enzymes responsible for hydroxylation reactions from any fungal source have yet to be fully purified and characterized, but are thought to be cytochrome P-450 dependent mono-oxygenases, whose mode of action involves the radical abstraction/rebound recombination process outlined in Figure 6 (Holland et al. 1990b). This has been confirmed by others, Griffiths et al. (1991). Although the isolation of membrane-bound cyt. P-450 monooxygenase from fungal sources is fraught with difficulty (Holland et al., 1990, Holland , et al., 1993, Griffiths et al, 1987), current research suggests the progesterone 11 α -hydroxylase system of *Rhizopus nigricans* is made up of four proteins: cytochrome P-450, NADPH-cytochrome P-450 reductase, cytochrome b5, and cytochrome b5 reductase (Osborne, et al., 1989). All these four proteins have been shown to be located on the cytoplasmic side of the endoplasmic reticulum. The latter two are monotopic in that they are only partially buried within the lipid bilayer. The exact spatial relationship of the four proteins within the membrane is not known, but experimental evidence suggests that the protein molecules diffuse within the plane of the membrane and interact by collision-coupling to form transient dimers and even ternary complexes. Taniguchi et al. (1984) showed that the substrate binding site of membrane-bound cytochrome P-450 directly faces the vertical plane of the lipid bilayer. This is important, since most substrates are lipophilic and will partition into the membranes, giving a higher local concentration than in the surrounding cytosol. The

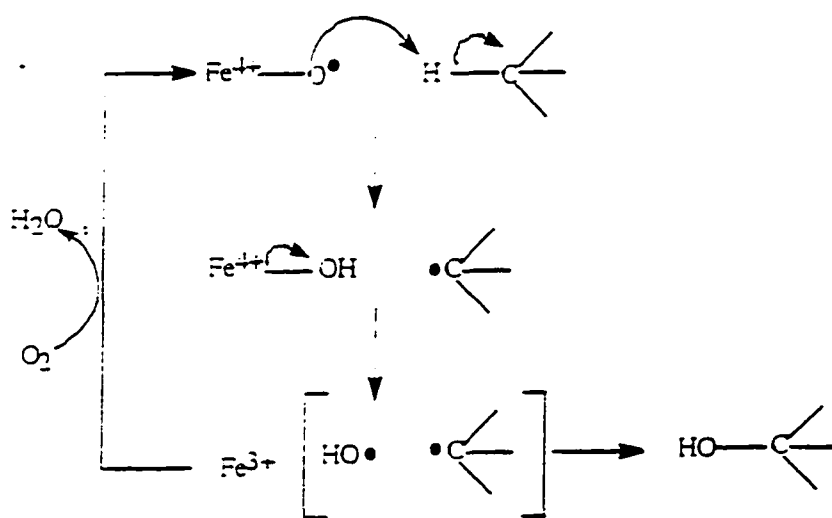


Fig. 6. Catalytic cycle of cytochrome P-450 dependent mono-oxygenases.

cofactor NADPH is oxidized in the reaction and needs to be continually recycled to sustain the conversion.

5. Stereochemistry of microbial hydroxylation

Most microbial hydroxylations show stereospecificity and/or stereoselectivity. The former term refers to the microorganisms or enzymes which only produce one kind of enantiomer of the compound, while stereoselectivity refers to those microorganisms or enzymes which only select one kind of stereoisomer as substrate if a racemic form is given. They may have regio- and enantioselectivity. As mentioned previously, specificity and selectivity are advantages that microbial biotransformation has over chemical synthetically methods. That limonene is degraded through four pathways by different microorganisms is an example of both stereoselectivity and stereospecificity.

Fungal hydroxylations of cyclohexene by *Aspergillus niger* give stereospecific products (Bhattacharyya and Ganapathy, 1965). Enantioselectivity of metabolism of some monoterpenes by *Armillariella mellea* (honey fungus) was studied by Lusiak and Siewinski (1989). (\pm) α -Pinene oxidized to (4S)-*trans*-sobrerol faster than to the (4R)-isomer. (4R)-*Trans*-sobrerol and (4S)-1,2,8-p-menthantriol were formed in excess from (\pm)- α -terpineol, whilst transformation of (\pm)-limonene yielded an excess of (4R) 8-p-menthene-1,2-diol. Braddock and Cadwallader (1995) reported that α -terpienol dehydratase (α TD) stereospecifically converted (4R)-(+)-limonene to (4R)-(+)- α -terpineol or (4S)-(-)-limonene to (4S)-(-)- α -terpineol. This enzyme might also be described as a Δ -8,9-limonene hydratase. α TD also showed stereoselectivity, since the

hydration rate of (4R)-(+)-limonene was approximately 10 times the hydration rate of (4S)-(-)-limonene.

Microbial hydroxylations at the enolizable C-2 and C-6 of Δ^4 -3-ketosteroids produce only axial (β) products: the corresponding equatorial (α) hydroxylations are not available microbiologically (Holland et al., 1975). However, the Δ^4 -3-ketosteroid analog (\pm)-4a-methyl-4,4a,5,6,7,8-hexahydro-2(3H)-naphthalenone is hydroxylated at both C-8 α and C-8 β by *Rhizopus arrhizus* ATCC 11145. The hydroxylation of 13-ethyl-gon-4-ene-3,17-dione to 15 α -hydroxylated product by *Penicillium raistrickii* was also found to be stereoselective (Schlosser and Schmauder, 1991).

Stereochemical side chain hydroxylations of aromatic compounds by fungi were reported by Holland et al. (1986). Three fungi were tested against 30 different aromatic compounds. The stereochemical properties were fungal species and substrate specific. However, the R absolute configuration of product predominated in most cases. It is believed that monooxygenases were involved in these hydroxylations. The stereochemistry of product formation is presumably controlled by the interaction between a benzylic radical intermediate and the oxygenating species. The size of the substituents may also play a role.

Holland et al (1993) elucidated the topography of the hydroxylase enzyme active site from *Mortierella isabellina*, which carried out the benzylic hydroxylation of toluene and related compounds. The enzyme showed substrate stereoselectivity based on the nature, position and size of substituent side chains close to the site of hydroxylation. Azerad (1993) reported regio- and enantioselective hydroxylation patterns of selected

bi(poly)cyclic enones by *Mucor plumbeus*. Enantiomeric excess (ee) was calculated by the amount of excessed enantiomer to total amount of both enantiomers (Allegrone et al., 1991), from 6-62% of α - or β -hydroxylation were found.

According to Yang (1988), cytochrome P-450 isozymes from animal system also shows stereochemical properties during hydroxylation and epoxidation. Cytochrome P-450 isozymes contained in various liver microsomal preparations have varying degrees of stereoselectivity in catalyzing the epoxidation reactions at various formal double bonds of polycyclic aromatic hydrocarbons. It was concluded that cytochrome P-450c, the major cytochrome P-450 isozyme contained in liver microsomes from rats, has the highest degree of stereoselectivity.

IV. Fungal immobilization

Using immobilized microbial cells for biotransformations has the advantage (Fukui and Tanaka, 1982) of freedom from enzyme extraction and purification, higher operational stability, greater potential for multi-step process, and greater resistance to environmental perturbations. Three major techniques are available for the immobilization of microbial cells, carrier binding, cross-linking and entrapment (Cibata, 1978, Faber, 1992, and Mahato and Majumdar, 1993). Entrapment of cells is the simplest method for cell immobilization and is carried out by encapsulation in gels or polymer networks or by immobilization in membranes (Faber, 1992). Immobilization of fungi has been reported for far fewer instances than has that of bacteria or yeasts (Koshcheyenko et al., 1983 and Ceen et al., 1987). The mycelial morphology complicates the initial immobilization and

any subsequent growth is more difficult to accommodate within a support structure (Fukui and Tanaka, 1984).

So far, few fungal immobilizations have been reported for preparations of flavor compounds even though numerous bioconversions have been done using free cells (Janssens et al., 1992). The microbial conversion of β -ionone to a mixture of its derivatives, that is utilized as an essential oil additive of tobacco, with immobilized *Aspergillus niger* was reported by Sode et al. (1989). *A. niger* was repeatedly used for microbial conversion of β -ionone in the presence of isooctane. Steroid hydroxylation using immobilized spores of *Curvularia lunata* germinated in situ was investigated by Ohlson et al. (1980). The fungal spores were immobilized in polyacrylamide granules or in calcium alginate beads (2-3 mm in diam.). The beads were used for the biotransformation of cortexolone to cortisol by steroid-11 β -hydroxylation. It was found that preparations based on calcium alginate gave the best results. In another study, *Rhizopus stolonifer* mycelia was gel-entrapped and used for production of 11 α -hydroxylation of progesterone (Sonomoto, et al., 1982). Ceen et al. (1987) immobilized *Aspergillus ochraceus* mycelia using alginate for production of 11 α -hydroxylation of progesterone in organic solvents. Fungal spores of *Rhizopus nigricans* NCIM 880 immobilized by Vidyarthi and Nagar (1994) in polyacrylamide, agar and chitosan matrices, were tested for their ability to produce the same product. They found that the active reusable biocatalyst beads, had faster rates of hydroxylation and produced higher yields of product. Schlosser et al. (1993) used immobilized spores of *Penicillium raistrickii* for 15 α -hydroxylation of 13-ethyl-gon-4-en-3,17-dione in the presence of β -

cyclodextrin. The product formation of both free and immobilized cells was increased in the presence of β -cyclodextrin, in comparison with reactions carried out with the presence of methanol.

Immobilized fungi have been used for production of some organic acids, e.g. citric acid and extracellular enzymes. Production of citric acid with immobilized *Aspergillus niger* was reported by Eikmeier and Rehm (1984). Cellulose was used as a carrier for immobilization of *A. niger* for production of citric acid by Fujii et al. (1994). The authors investigated different volume ratios of the carrier for citric acid production. The results showed that the acid productivity in the immobilized culture was twice that in a conventional suspension culture. It was found that the acid productivity depended on carrier volume ratio, where a maximum productivity of 8.9 g/l/d was obtained at 30% v/v.

Immobilized growing cells of *Gibberella fujikuroi* P-3 were used for production of gibberellic acid and pigments in batch and semi-continuous cultures (Kumar and Lonsane, 1988). The performance of this immobilized fungi was affected by immobilization agent, nature and age of cells, mycelial cell density, size of beads and inclusion of linseed oil. Kutney et al. (1988) reported biotransformation of dehydroabietic, abietic, and isopimaric acids by *Mortierella isabellina* immobilized in polyurethane foam. The purpose of the study was to find the system which can be used for detoxifying resin acids found in certain pulp mill effluents. They found that optimal dehydroabietic acid transformation occurs with early-stationary-phase foam-bound mycelia suspended in buffer at pH 6.5 to 8.5 with aeration $>0.1 \text{ liter liter}^{-1} \text{ min}^{-1}$ and near a temperature maximum of 33°C.

There is increasing interest in the application of immobilized growing mycelial cell systems for enzyme production. Most extracellular enzymes obtained by this method have been produced by gel-entrapped fungal cells (Bon and Webb, 1989; Bailey and Poutanen, 1989). Bon and Webb (1989) reported glucoamylase production by immobilization of *Aspergillus awamori* spores. Manolov (1992) reported ribonuclease production by immobilized *Aspergillus clavatus* cells in a bubble-column bioreactor. Ribonuclease production has been studied under batch, repeated-batch and continuous fermentation conditions in the bioreactor system and compared with production by free cells. Enzyme production by immobilized cells (IC) during batch fermentation was comparable to that of a free-cell system. The specific productivity of IC was 8.5 times higher than that of free cells. Continuous ribonuclease production was achieved for 44 days at 1 aeration volume per volume per minute (vvm) and a dilution rate of 0.01 per hour of the volume (h^{-1}) with high volumetric productivity ($450 \text{ U.l}^{-1}.\text{h}^{-1}$) and yield.

V. Whole-cell bioconversions in aqueous-organic solvents systems

1. Use of organic solvents in whole-cell biotransformations

An organic solvent may be the substrate of interest, or it may be used to shift the equilibrium in a favorable direction. Many of the flavor substrates are essentially insoluble in aqueous media and/or are highly toxic to whole cells (Lanne et al., 1987a; Andersson and Hahn-Hagerdal, 1990; Sonsbeek et al., 1993; Halling, 1994; Salter and Kell, 1995). The advantages of using organic solvents to aid biotransformations by viable

cells are given in Table 4. However, there are also a number of potential disadvantages to consider when using organic solvents, these include those tabulated in Table 5.

2. Solvent selection for whole cell biotransformation

The most significant problem in using organic solvents with viable cells lies not with the system or reactor employed, but rather in the choice of solvent. Many solvents are highly cytotoxic or inhibitory. Those that are nontoxic have restricted solvating power and are consequently of limited use as a solvent. This problem is compounded by the fact that different cell types, lines, or their individual strains may vary considerably in their response to a given solvent, even under the same physiological conditions (Salter and Kell, 1995). Thus, selection of solvent is a determining factor for organic media biotransformation.

Three types of organic media: single-phase; two-phase; and reversed micelles have been reported. True single-phase systems, are produced when water-miscible cosolvents are added to the medium to improve the solubility of compounds that are relatively insoluble in aqueous systems (Salter and Kell, 1995). This can reduce considerably the mass-transfer limitations, resulting in more rapid reaction rates. Two-phase systems consist of a continuous and a discontinuous phase, formed by two (or more) immiscible liquids. The aqueous phase will contain the biocatalyst either dissolved or in colloidal or insoluble form (quite possibly immobilized); hence, it is sometimes known as the “biophase”. Organic solvents are in nonaqueous-phase, including the substrate/product, typically in low concentrations. Reversed micelles are also called microemulsions. These are thermodynamically stable, “single-phase” systems, wherein the

Table 4.
Potential advantages of the use of organic solvents in
whole-cell biotransformations

They can increase the concentration of poorly water-soluble substrate/products
They can reduce product and/or substrate inhibition
They can prevent hydrolysis of substrates/products
Many organic solvents are themselves of interest as substrates
There may be a reduction of mass-transfer limitations
They may alter the partitioning of the substrate/product
They may improve the stereoselectivity of a biotransformation
They may improve the ease of product recovery
Their use may allow a better integration with chemical steps/processes

(Following Salter and Kell, 1995)

Table 5.
Potential disadvantages of the use of organic solvents in
whole-cell biotransformations

The solvent may be cytotoxic/inhibitory to the biocatalyst (and to other life forms)
Nontoxic compounds tend to be highly apolar and have poor solvation properties and low reaction rates
Reactant complexes that are poorly soluble in both aqueous- and organic-phase may precipitate out at the interface
There is an increase in system complexity, which is always undesirable
Costs will increase to ensure safety, both within the reactor and downstream
There is necessarily a problem of waste disposal, or at least of recycling
Very little real experience exists to draw upon, especially on a large scale
Product recovery may be problematic, especially if surfactants are used and/or emulsions are formed

(Following Salter and Kell, 1995)

addition of an appropriate amphiphile or detergent (surfactant) permits the single-phase coexistence of otherwise mutually insoluble aqueous and organic media.

There are two questions that need be answered when choosing organic systems. First, which solvents can be used. Second, what concentration is safe to use. The answer of both questions is based on the toxicity of the solvents to the microorganisms. With regard to the nature of the organic solvent used (Bruce and Daugulis, 1991), a widespread consensus has emerged, based on many studies with single solvents, that, to a reasonable approximation, biocatalyst stability with respect to log P, logarithm of the octanol:water partition coefficient of the solvent, decreases as log P increases, reaching a minimum at log P values of 0 to 2 for enzymes and 2 to 4 for microorganisms, after which increasing log P of the solvent (or for that matter substrate) results in increased biocatalyst stability (Lanne et al, 1987a,b; Hocknull and Lilly, 1987; Inoue and Horikoshi, 1991; Osborne et al., 1992), i.e., biocatalysts are more stable in less polar solvents. The transition from toxic to nontoxic solvents typically occurs between log P 3 to 5, and depends on the homologous series (Vermue et al., 1993). Concentration for cytotoxicity is almost as important as the kind of solvents to be chosen. Using dielectric spectroscopy to assess cytotoxicity, it was found that the majority of organic solvents tested demonstrated a "threshold effect", in that there is a quite specific concentration at which the solvent becomes toxic. Small increases in the concentration above this threshold had a marked effect on cell viability (Salter and Kell, 1995).

Immobilization of cells is a way to overcome some problems seen using free cells in organic solvent systems, such as inactivation by the solvent, precipitation of the

biomass, and the aggregation of cells at the liquid-liquid interface (Brink and Tramper, 1985). Microorganisms may partition/accumulate at the liquid-liquid interface when two-phase systems are used. Rosenberg et al. (1980) demonstrated that various bacterial strains thought to possess hydrophobic surface characteristics adhered to liquid hydrocarbons such as hexadecane, octane, and xylene. This is the basis of the MATH (microbial adhesion to hydrocarbons, formerly BATH, the bacterial adhesion to hydrocarbons) assay, a test for measuring cell-surface hydrophobicity. This property also helps to select hydrophobic solvents in two-phase systems. There is the possibility of using a two-phase system wherein the cells are immobilized/adhered directly onto the organic/hydrocarbon phase, which in turn can carry the substrate of interest. For such a system to be successful, both the organic solvents/hydrocarbons used need to be nontoxic (Salter and Kell, 1995).

3. Biotransformations on aqueous two-phase systems

Many studies have been published on microbial biotransformation in aqueous two-phase systems. A water insoluble substance, β -sitosterol, was transformed to ADD (1,4-androstadiene-3,17-dione) and AD (4-androsten-3,17-dione) by immobilized *Mycobacterium fortuitum* (DSM 1134) in organic media (Steinert et al., 1987). Both hydrophilic (alginate and chitosan) and hydrophobic (silicone and polyurethane) matrices were tested. They found that in a two phase system of a bulk phase and an aqueous catalyst phase, the solution power and the low toxicity of the bulk phase solvent is more important than the matrix on the distribution coefficient. Ceen et al. (1987) found that immobilizing *Aspergillus ochraceus* reduced conventional solvent damage, compared

with free cells, for production of 11α -hydroxylation of progesterone. The authors also showed that use of natural oils as solvents enhanced biotransformation performance. After studying the effects of the hydrophilicity and hydrophobicity and the network structures of gels entrapping biocatalysts for bioprocesses in organic solvent media, Fukui et al. (1987) concluded that use of hydrophobic gels and less polar solvents was preferable for bioconversions of lipophilic compounds. Boeren et al. (1987) studied the viability and activity of *Flavobacterium dehydrogenans* in two-liquid-phase-systems. The steroid conversion catalyzed by the bacterium was from androstenedione (Aac) to 4-androstene-3,17-dione (AD). The results showed that (1) the conversion rate in these two-liquid-phase systems were significantly higher than in aqueous media if the growth stage of the microorganism at the moment of substrate and organic solvent addition was chosen properly; (2) viability was high even in alkane-substituted solvents with a hydrophobicity between $\log P$ 2 and $\log P$ 4.

The bioconversion of vanillin to vanillyl alcohol in a two-phase reactor using alginate immobilized yeast cells of *Saccharomyces cerevisiae* was investigated by Wulf and Thonart (1989). Several solvents were screened for toxicity and bioconversion inhibition assessment. Only dodecanol gave good cell viability and good bioconversion yields. Several parameters, such as, volume ratio of aqueous over organic phase, pH, vanillin concentration influenced the bioconversion. After screening 11 solvents, Sode et al. (1989) used isooctane as an organic solvent to improve microbial conversion of β -ionone by immobilized *Aspergillus niger*. The addition of isooctane accelerated the microbial conversion of β -ionone two folds. The addition of isooctane improved the

resistance of *A. niger* to the antifungal properties of β -ionone. Another solvent, n-decane-1-ol, was reported to improve Δ^1 -dehydrogenation of steroids (6- α -methyl-hydrocortisone-21-acetate) by immobilized *Arthrobacter simplex* (Pinheiro and Cabral, 1992). The same group (Fernandes et al., 1995) assayed chloroform, toluene, and n-octan-1-ol as organic solvents for bioconversion using the same microorganism. They found that n-octan-1-ol was the most appropriate solvent for the steroid solubility and biocompatibility. Addition of surfactants (a commercial-grade lecithin, phosphatidylcholine, and phosphatidylethanolamine) led to increased initial reaction rates but did not significantly change the final conversion yields.

VI. This study

This study was designed to develop a system using *P. digitatum* (NRRL 1202) for the conversion of limonene to a valuable flavor component, α -terpineol. Currently marketed α -terpineol is produced by hydration of pinene or turpentine oil using mineral acids. α -Terpineol production through biotransformation may be labeled as natural product. Increasing consumer preference for food products containing “natural” flavors over those containing artificial (synthetic) flavors has led to an increase demand for natural flavor and aroma components. Properties of this bioconversion were investigated and optimum conditions were determined. An immobilized fungal cell reactor was designed and operated for production of α -terpineol at batch, repeated batch and continuous feed methods.

MATERIALS AND METHODS

I. Chemicals

(R)-(+)- and (S)-(-)-limonene and an authentic sample of α -terpineol were purchased from the Aldrich Chemical Company (Milwaukee, WI., USA). The purities of (R)-(+)- and (S)-(-)-limonene were 97% and 95%, respectively; α -terpineol was 98% pure. These chemicals were further purified by passing them through a silica gel column. Eluents of purified terpenes were collected. The purity of these terpenes, as determined individually by gas liquid chromatography (GC), were (R)-, (S)-limonene and α -terpineol, 98.8, 96.1, and 99.3% respectively. Sodium alginate, low viscosity, used for fungal immobilization was purchased from the Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals or solvents were of the best available commercial grade.

II. Organism and growth conditions

Twenty different fungal strains were screened prior to this investigation. The screening procedures followed steps outlined in Figure 2. After fungi reached growth phase, 0.1% of limonene was added to the culture which was maintained on the shaker at 28°C for 2 days. The bioconversion products were extracted with 5 ml of diethyl ether. Extractants were concentrated to 0.5 ml under nitrogen stream before they were injected into GC-MS for product identification. *Penicillium digitatum* (NRRL 1202) was selected because it was the only fungus of those tested that showed ability to convert limonene to α -terpineol. This organism was maintained on potato dextrose agar (PDA, Difco) slants. After sporulation, stock cultures were stored at 4°C. Spores were produced for culture

inocula from PDA plates inoculated from stock culture slants. These PDA plates were maintained at 25°C for 2 weeks prior to use as spore inoculum. The growth medium was that of Abraham et al. (1986). This medium contained 10 g glucose/L of, 10 g peptone/L, 20 g malt extract/L, and 3 g yeast extract/L. The pH of the medium was adjusted to pH 7.0 with NaOH (40%, w/v) prior to sterilization. Spores were transferred aseptically into 250 ml Erlenmeyer flasks containing 50 ml of growth medium and incubated for 12 hrs. Then, the culture was transferred to fresh medium in 1:10 (v/v) ratio for another 12 hrs incubation prior to use for in bioconversion studies. The cultures were grown on a rotary shaker (NBS Model G25-KC rotary shaker, NBS Co., Edison , NJ) at 28°C and 100 rpm.

III. Growth and bioconversion activity

Bioconversion by both growing cells and resting cells was investigated. Bioconversion activity with growing cells was tested using new and aged inocula. The new inoculum was 1-day old germinating spores, and aged inoculum was 6-days old growing mycelia. For determination of bioconversion activity, five ml of growing fungal mycelial culture was aseptically withdrawn every 12 hrs. To each 5 ml sample, fifty µl of limonene was added and the mixture vortexed for 30 sec. It was then incubated on a shaker for 12 hr at 100 rpm and 28°C prior to extraction with diethyl ether. At each sample time, 10 ml of the culture was also taken for a dry weight determination. Fungal mycelia were removed by filtration through a No.1 Whatman filter paper and then dried to constant weight at 110°C.

Resting cells were produced from a 12 hr fungal mycelial culture. Two hundred ml of 12 hr culture were harvested by vacuum filtration using a No.1 Whatman filter paper. Weighed mycelia (2.4 grams, wet) were then resuspended into 200 ml of sterile 0.05M pH 7.0 citrate-phosphate buffer. Two ml of limonene were then added to the flask, which was incubated on a shaker at 28°C. Five ml samples were withdrawn at 12 hrs intervals. Each sample was extracted with 5 ml of redistilled diethyl ether. An internal standards solution (50 μ l) containing two internal standards (tetradecane and 1-decanol) was added to each reaction mixture. Tetradecane and 1-decanol were internal standards (I.S.) for limonene and α -terpineol (α T) respectively. Both chemicals were prepared as 5000 ppm stock solutions in methanol. For sample extraction, five ml of ether was added to each reaction tubes, containing 5 ml of reaction mixture with the internal standards. The reaction mixtures were vortexed for 30 sec. Anhydrous sodium sulfate (4g) was added to break the emulsion. The ether fraction was separated, dried over anhydrous Na_2SO_4 , and then concentrated under a stream of nitrogen to 1 ml, prior to analysis by GC.

IV. Analytical methods

Compounds in the ether extract were quantified by GC/MS. The GC/MS system consisted of an HP 5790A GC/HP 5970B mass selective detector (MSD) (Hewlett Packard Co., Palo Alto, CA). Each extract (5 μ l) was injected in the splitless mode (250°C injection temperature) into a fused silica open-tubular (FSOT) capillary column (Superlcowax 10, 60 m length x 0.25 mm i.d. x 0.25 μ m film thickness; Superlco, Inc.,

Bellefonte, PA). Helium (purity 99.999%, passed through hydrocarbon trap, molecular sieve, and an oxygen trap) was used as the carrier gas at a linear velocity of 25 cm/sec. The oven temperature was programmed from 40°C to 200°C at 6°C/min with an initial hold time of 5 min and a final hold time of 50 min. Splitless valve time was maintained for 30 sec. MSD conditions were as follows: capillary direct interface temperature, 200°C; ionization voltage, 70 eV; mass range, 33-300 a.m.u.; electron multiplier voltage, 1800 V; and scan rate, 1.60 sec⁻¹.

Positive identifications were confirmed by matching sample retention indices (RI), calculated according to Van den Kratz (1963), and mass spectra of samples with those of authentic standards analyzed under identical experimental conditions. Tentative identifications were based on the standard Wiley/NBS library data (Hewlett Packard, 1988).

After α -terpineol was identified as a product of the *P. digitatum* conversion of limonene, samples were routinely analyzed using an HP 5890 GC with flame ionization detector (FID) (Hewlett Packard Co., Palo Alto, CA). Sample extraction and preparation was the same as above. This GC was equipped with a FSOT column (Superlcowax 10, 60 meters length x 0.32 mm i.d x 0.25 μ m film thickness; Superlco, Inc., Bellefonte, PA). Helium was used at a linear velocity of 22 cm/sec. Oven temperature was programmed isothermally at 180°C. Five μ l was injected into the column with the split mode (split ratio 1:30). Injector and detector temperatures were 225°C and 250°C, respectively.

The concentrations of limonene and α -terpineol in each extract were quantified from calibration curves for each using peak area ratio's (analyte/I.S.) vs amount ratios (analyte/I.S.) with standard authentic samples under identical conditions. The following equations were obtained from standard curves for calculation of α -terpineol (α T) and limonene in each sample.

$$\alpha\text{-Terpineol in sample (mg/5 ml)} = [(Area \alpha T/area IS \text{ in extract}) - 0.104] \div 4.372 \\ * 0.807;$$

$$\text{Limonene (mg/5 ml)} = [(Area limonene/area IS \text{ in extract}) + 0.019] \div 4.132 * \\ 0.832.$$

where 0.807 and 0.832 were the relative recovery factors for α -terpineol and limonene, respectively (see V of this section). Bioconversion extent, which was expressed as a percentage, and used to determine the bioconversion efficiency, was calculated from the ratio of α -terpineol produced to the amount of limonene added.

V. Extraction recovery

The percentage recovery by diethyl ether liquid-liquid extraction was determined by using five known amounts of authentic α -terpineol and limonene added into fresh medium, cell broth, and immobilized cell reaction medium (0.05 M, pH 7 citrate phosphate buffer). The standard samples were extracted with ether in a manner identical to test samples. The amount of extracted α -terpineol and limonene were determined as described above. This test was done in triplicate and the average percentage recovery

calculated. The reciprocal of the recovery was used as the factor for determining the original amount α -terpineol and limonene in samples.

VI. Substrate induction

Single addition substrate induction was conducted by spiking 200 ppm of limonene into growing cells cultures at one of three different growing stages (early-log, mid-log, and stationary phase) and continuous induction was performed by spiking 200 ppm limonene at each of these three growth stages. The culture broth without limonene spiking was used as a control. Bioconversion activity for the control and induced cells was measured by the methods described previously.

VII. Enzyme inhibition

Five different concentrations of authentic α -terpineol (0, 6.5, 13.0, 19.5, and 26.0 mM) were added into 5 ml samples of growing cell culture (log phase, 36 hrs growth, 4.96 mg/ml dry weight cells) to determine α -terpineol inhibition. Absence of α -terpineol was the control. Limonene (1%, v/v) was added as substrate. Bioconversion procedures, sample extract and analyses were the same as previously described. The bioconversion activity or velocity was obtained under each α -terpineol concentration. The K_{iapp} of α -terpineol as an inhibitor can be calculated from the plot of α -terpineol concentration versus newly formed product (total amount from analysis minus amount addition) at each concentration.

Many fungal hydroxylation reactions are catalyzed by a cytochrome P-450 monooxygenase system. This enzymatic system contains flavoproteins and may be sensitive to iron chelating agents. Six different concentrations of phenanthroline, from 10^{-6} to 10^{-3} M, were tested on bioconversion of both free and immobilized cells. Procedures were as described above. The K_i 's for both free and immobilized cells can be determined by plotting of the activity under each concentration of the inhibitor.

VIII. Determination of the Michaelis-Menten constant

The approximate Michaelis-Menten constant [$K_m(\text{app.})$] was determined by the method of Lineweaver and Burk (1934). Eight different concentrations of limonene (0.0062, 0.0123, 0.0308, 0.0617, 0.0925, 0.1233, 0.1541, 0.3083 mM) were tested for both free cells and immobilized cells. Free cells were five ml of 12 hr. growing cells (0.0113 g dry cells), and immobilized cells were 4 grams of calcium alginate beads (0.0109 g dry cells) in 5 ml of 0.01 M pH 7 citrate-phosphate buffer. Activity of each bioconversion was assayed for 12 hrs under a 28°C and 100 rpm shaker. Bioconversion products were extracted and analyzed as previously described. The $K_{m\text{app}}$ of free and immobilized cells were obtained by plotting reciprocal activities versus reciprocal limonene concentrations.

IX. Determination of product stereochemistry

Most natural limonene is (R)-(+)-limonene. In order to determine the stereospecificity and/or stereoselectivity of the bioconversion process, conversion of both

(R)-(+)- and (S)-(-)-limonene were tested. The ether extraction fractions were analyzed by a chiral GC. Each extract (5 μ l) was injected in the split mode (225°C injector temperature) into SGE 7959 fused silica capillary column (Cydex-B, 50 m length x 0.22 mm i.d. x 0.25 μ l film thickness; SGE Scientific Pty. Ltd., Australia) on an HP 5890 GC. Helium was used as carrier gas at a linear velocity of 30 cm/sec. Oven temperature was 80°C, isothermal, for separation of limonene enantiomers and 120°C, isothermal, for detection of α -terpineol enantiomers.

X. Immobilization of *P. digitatum* on calcium alginate

Thirty ml of germinated *P. digitatum* spores (early log phase, 6 hrs growth) containing 24.5 mg/ml dry weight of fungi were transferred into 200 ml of fresh medium (see above) and then allow to grow another 6 hrs at 100 rpm and 28°C. This culture broth was aseptically suspended, into 230 ml of 10% (w/v) sodium alginate dissolved in the same medium to produce a 5% (w/v) of sodium alginate in the final mixture. The mixture was stirred and then pumped dropwise through a 0.01 inch diameter tube into 400 ml of cold 0.2 M of CaCl₂. After the beads formed, they were stored in the CaCl₂ solution for an hour to age. The beads were then removed by filtration and washed twice with 0.9% of sterile NaCl solution; once with sterile water; and twice with sterile 0.01 M pH 7 citrate-phosphate buffer. They were stored at 4°C until used.

XI. Separation of the calcium alginate beads from cell mass

Five grams of alginate beads were added to 50 ml of 0.1 M of phosphate buffer at pH 7.0 with continuous stirring. After 1 hour, the beads completely dissolved. The fungal biomass was separated from the mixture by filtration. The biomass was determined by drying in the oven to constant dry weight.

XII. Calcium alginate beads storage stability

Three gram samples of beads were weighed into test tubes and stored at -20 °C and 4°C. The bead bioconversion activity was tested at 0, 2, 4, 6, 8, 10, 12, and 14 days. Each bioconversion test was done by adding 3 ml of 0.01 M of pH 7.0 citrate-phosphate buffer and 50 µl of limonene into the test tube, and incubating for 1 day at 100 rpm and 28°C prior to extraction. The extract and analysis methods were the same as described above.

XIII. Optimization of limonene bioconversion

1. Aeration

The bioconversion of limonene to α -terpineol was found to require oxygen. Different levels of aeration were tested for their effect on this bioconversion for free and immobilized cells. The 50 ml bioconversion mixtures in 250 ml-shaking flask were placed at 0 rpm (standing), 100 rpm, and 200 rpm incubators with 28 °C. The actual amounts of dissolved O₂ in each flask were measured using a dissolved oxygen meter (

YSI model 58 with YSI 5730 dissolved oxygen probe, Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio).

2. Temperature

Both free cells and immobilized cells were tested for bioconversion activity at temperatures ranging from 24 to 40°C. In the case of free cells, the test was conducted by using free cell culture up to 36 hrs old (see section III), for immobilized beads, beads stored 1 to 7 days at 4°C were used. The bioconversion activity of both growing and immobilized cells of each treatment temperature (24, 28, 32, 36, 38, and 40°C) was paired with the same conditioned free or immobilized cells, which was treated at 30°C. Thus, the activity for each temperature was compared with that at 30°C (as a reference). The relative activities compared to the activity at a temperature of 30°C were calculated and the optimum temperature was determined.

3. pH

Citrate-phosphate buffers (0.05M) were used to cover the pH range from 2.5 to 8.0. Glycine-NaOH buffers (0.05 M) were used from pH 8.5 to 10.5. HCl (0.1 M) solution was used for pH 1. For free-cell pH optimum, resting cells (about 0.21g wet cells for each test tube), which were harvested from 10 ml of 1 day growing cells, were used. Five ml of a pH buffer were added to the appropriate test tube containing resting cells. Three grams of immobilized cells were suspended into 3 ml of each buffer for the immobilized cells test. Fifty µl of limonene as the substrate was added into each test tube. The final pH was recorded before each sample extracted with ether. Bioconversion procedure and sample analyses of these tests were as described previously.

4. Limonene concentration

Ten different limonene concentrations ranging from 0.02 to 8% (v/v), in 5 ml reaction mixtures, were tested with growing cells (36 hrs growth culture) to determine optimum substrate concentration of free cell bioconversion. For immobilized cells, concentrations ranging from 0.03 to 1.6% (v/v) were added into 3 g beads suspended in 3 ml of 0.05 M, pH 7, citrate-phosphate buffer. Bioconversion activity at each concentration was obtained as described previously.

5. Contact time

In order to achieve the highest yield and prevent degradation of α -terpineol by the fungus, it was important to know the optimum contact time with the substrate. Growing cells, resting cells, and immobilized cells were investigated for their bioconversion activity based on contact time (0 hr to 144 hrs). Samples were taken every 6-12 hr for product formation analysis.

XIV. Bioconversion of immobilized cells with batch and repeated batch form

Batch bioconversion, using immobilized cells, was conducted by suspending 30 grams of immobilized beads into a 250 ml flask containing 100 ml of 0.01 M pH 7.0 citrate-phosphate buffer. One percent of limonene (v/v) and 1% Tween 80 (v/v) were added to the flask which was then placed on the 28°C shaker. Five ml samples were taken every 24 hrs for product determination. After 4 days, the highest bioconversion activity was achieved. Batch bioconversion was then stopped. The beads used in the batch bioconversion were washed thoroughly prior to repeating the bioconversion. Repeated

batch bioconversion was carried out as follows. The beads, or mycelia were separated from the old reaction medium, washed three times with 0.9% (w/v) NaCl solution and twice with buffer before new 100 ml reaction medium (0.01 M pH 7.0 citrate-phosphate buffer with 1% of limonene (v/v) and 1% of Tween 80 (v/v)) was added. Five ml of samples from this batch again were withdrawn every 24 hrs for product determination as in the previous batch. This procedure was repeated until the activity significantly dropped. Then the beads were regenerated with growth medium for 3 days to allow formation of cofactors (e.g., NADPH). The beads were then reused. This procedure was repeated 5 times.

XV. Bioconversion of immobilized beads in a continuously fed air-lift bioreactor

A continuously fed air-lift bioreactor was used for the continuous bioconversion of limonene to α -terpineol. The reactor was a 580 ml Kontes Airlift Bioreactor (Kontes Life Science Products, Vineland, NJ) and containing 500 ml of the reaction medium at the start. One hundred and fifty grams of immobilized calcium alginate beads were loaded into the pre-sterilized bioreactor. The feed 1% (v/v) of limonene and 1% (v/v) of Tween 80 in sterile 0.01 M citrate-phosphate buffer (pH 7.0) was stirred continuously. The air-lift column was fed from the bottom. The air into the reactor was regulated with an in-line direct reading air flow meter (65-MM, Cole-Parmer Instrument Co., Niles, Ill). The air was pre-filtered through two sterile Whatman Hepa-vent filters (Whatman Inc., Clifton, NJ).

Two parameters were optimized, aeration and feed rate. Four aeration rates (0, 0.2, 0.3 and 0.4 Standard Liters Per Minute (SLPM)) were compared. During each run, the amount of dissolved O₂ consumed by immobilized cells inside the column was monitored by the D.O. difference between the feed and the elute. This was measured using a dissolved oxygen meter (YSI model 58 with YSI 5730 dissolved oxygen probe, Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). Two feed rates (0.007 and 0.014 h⁻¹, or 100 ml and 200 ml/day) were tested. Because different batches of beads may have different initial bioconversion activities, 30 g beads from the same batch as that in the bioreactor was used as a control. They were suspended into a 250 ml flask containing 100 ml of the same reaction medium as the feed material to the column, and maintained at the same temperature as the bioreactor. The α -terpineol productivity from 8 runs was compared based on the relative productivity to its control.

XVI. Bioconversion in organic solvent systems

1. Effect of organic solvents on bioconversion of limonene

Twenty-two different solvents were added into bioconversion reaction mixtures to screen for their effect on the *P. digitatum* bioconversion of limonene to α -terpineol. These solvents were: o-xylene, cyclohexane, p-cymen, ethyldecanoate, butylbenzoate, tetradecane, dimethylphthalate, methanol, ethanol, n-amyl alcohol, hexane, isooctane, dimethyl-sulfoxide, Tween 80, polyethylene glycol, propylene glycol, dipropylene glycol, tripropylene glycol, glycerol, Triton 100-x, dioctylphthalate. The relative activity was obtained by comparison to the control without solvent addition. Because immobilization

may change responses of the fungus to organic solvents, both growing cells and immobilized cells were tested with these solvents. The concentrations of these solvents were 1.5 to 2% (v/v) of the reaction mixtures during bioconversion process.

2. Optimization of dioctylphthalate and methanol concentration for free cell bioconversion

Dioctylphthalate at 1.5% was found to significantly improve the yield of α -terpineol produced by free cells. This organic co-solvent concentration was optimized for improvement of the bioconversion process. Seven different concentrations (0, 0.5, 1, 1.5, 2, 3, 4% , v/v) of dioctylphthalate were tested.

Methanol is often used to improve the reaction rate of the bioconversion of steroids (Sode et al., 1989). In this study, methanol (1.5%) was also found to improve α -terpineol production compared with the control with free cells in the screening tests. The optimum concentration of this solvent was determined. Seven different concentrations (0, 0.1, 0.5, 1, 2, 3, 4, 8%, v/v) of methanol in reaction mixture were investigated. Zero methanol addition was used as a control. α -Terpineol production from the methanol addition treatments was compared with the controls. The optimum methanol was determined by concentration at which the highest α -terpineol production was obtained. This test was repeated three times and the mean results were reported.

3. Optimization of Tween 80 concentration for immobilized cell bioconversion

Tween 80 was found to improve the bioconversion yield of limonene to α -terpineol with immobilized cells. The optimum Tween 80 concentration was determined

based on triplicate testing of different concentrations (0, 10, 100, 200, 250, 500, 1000, 5000, 10000, 20000, 40000 and 80000 ppm) in the reaction mixture.

RESULTS

I. Proof of bioconversion

Bioconversion products were recovered by an ethyl ether liquid-liquid extraction technique. The substrate (limonene) and product (α -terpineol) recovery is listed in Table 6. α -Terpineol in the ethyl ether extract was determined by GC/MS. A typical total ion chromatogram-mass spectra of extracted sample is shown in Figure 7. The authentic standard α -terpineol chromatogram is shown in Fig. 8 for comparison. A chromatogram of a sample without limonene addition shows no α -terpineol production (Fig. 9). Figure 7A illustrates that α -terpineol peak was found after bioconversion of limonene by the organism. Figure 7B shows a mass spectra of an α -terpineol peak, which was identical to the mass spectrum and retention time of an authentic α -terpineol sample (Fig.8AB). In the control extract, no α -terpineol was detected (Fig. 9A). This eliminates the possibility that the fungus biosynthesized α -terpineol but rather bioconverted limonene to α -terpineol.

II. Physiological studies

1. Growing cells and α -terpineol production

Initially, 9 different common fungal growth media were screened for growth of *P. digitatum* (NRRL 1202) (Table 7). The best medium for growth and bioconversion was that of Abraham et al. (1986). This medium was used for all further experiments. A maximum cell mass was obtained after 5 days in the shake flask culture, however,

Table 6.
Recovery percentage of extraction method*

Medium Types	Limonene Recovery (%)	α -Terpineol Recovery (%)
Growing cell broth	83.2 \pm 4.5%	80.7 \pm 3.1%
Resting cell culture	84.5 \pm 4.8%	82.4 \pm 3.9%
Immobilized Beads	84.3 \pm 4.1%	81.8 \pm 3.3%
Growth medium	90.6 \pm 3.9%	85.4 \pm 2.5%

* The results are averages of four replications \pm the standard deviation (SD).

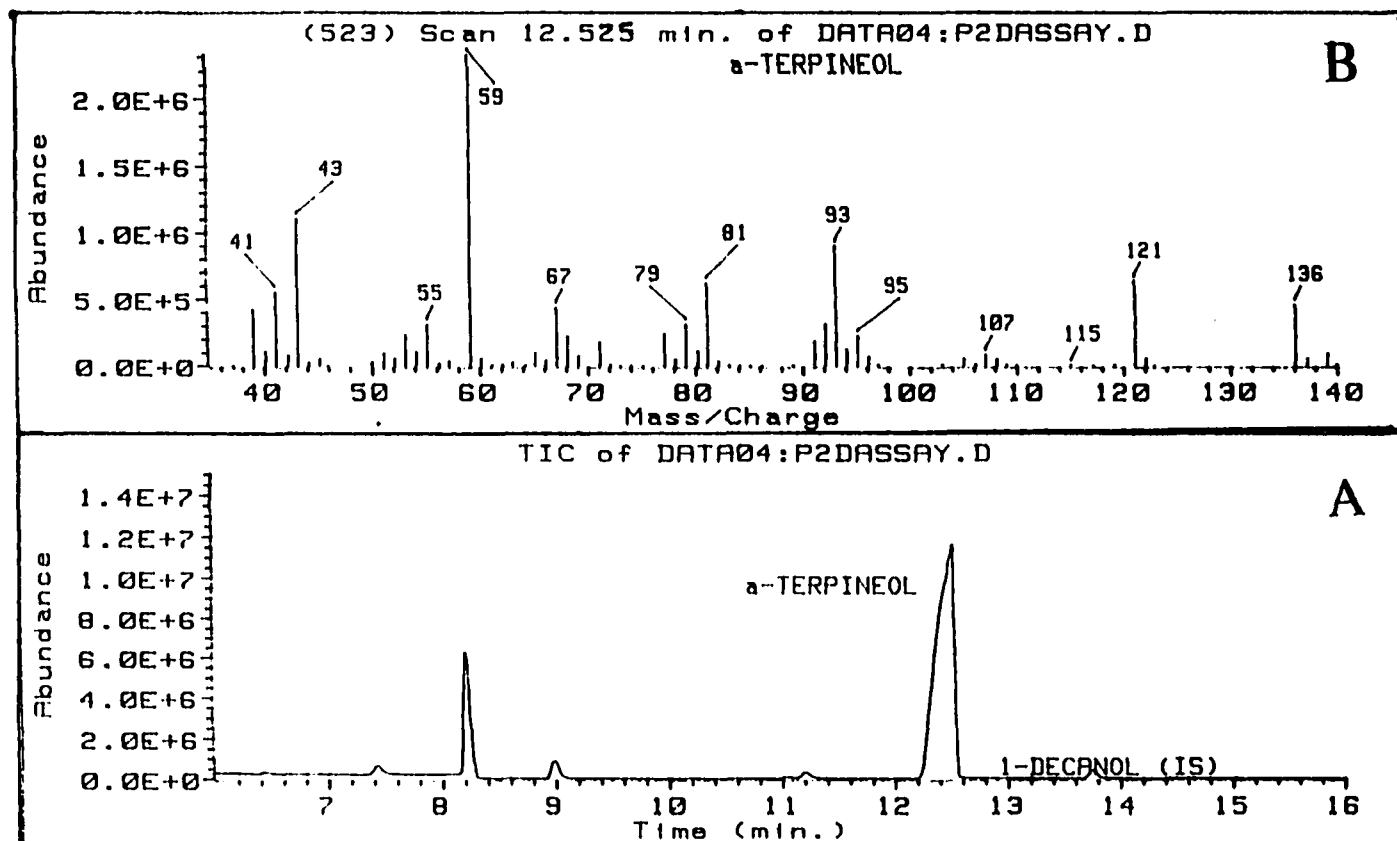


Fig. 7. Total ion chromatogram of *P. digitatum* bioconversion products extract after 2 days contact with substrate limonene (A). Mass spectrum at retention time 12.52 min (B).

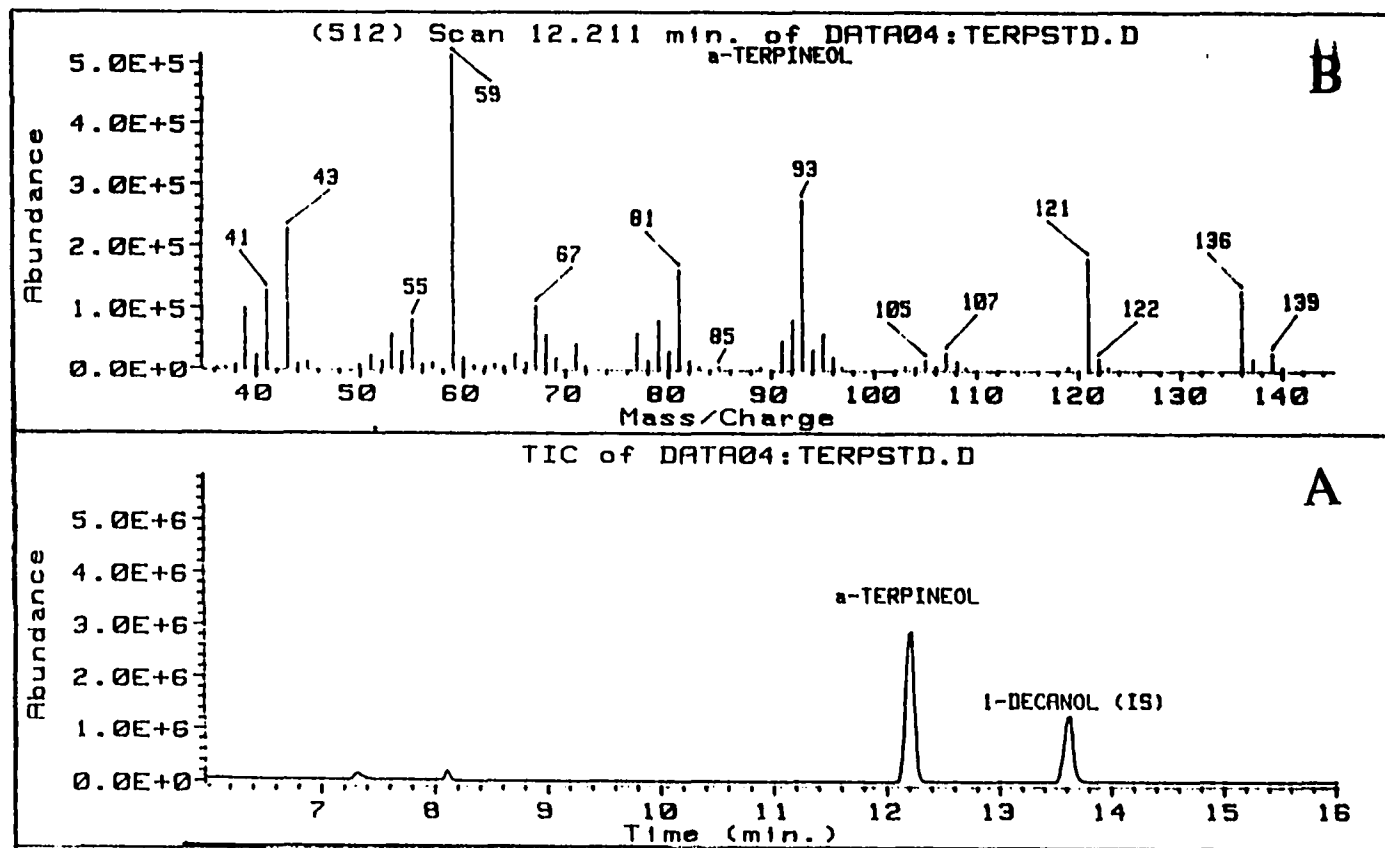


Fig. 8. Total ion chromatogram of a standard mixture of α -terpineol and internal standard 1-decanol (A). Mass spectrum at retention time 12.21 min (B).

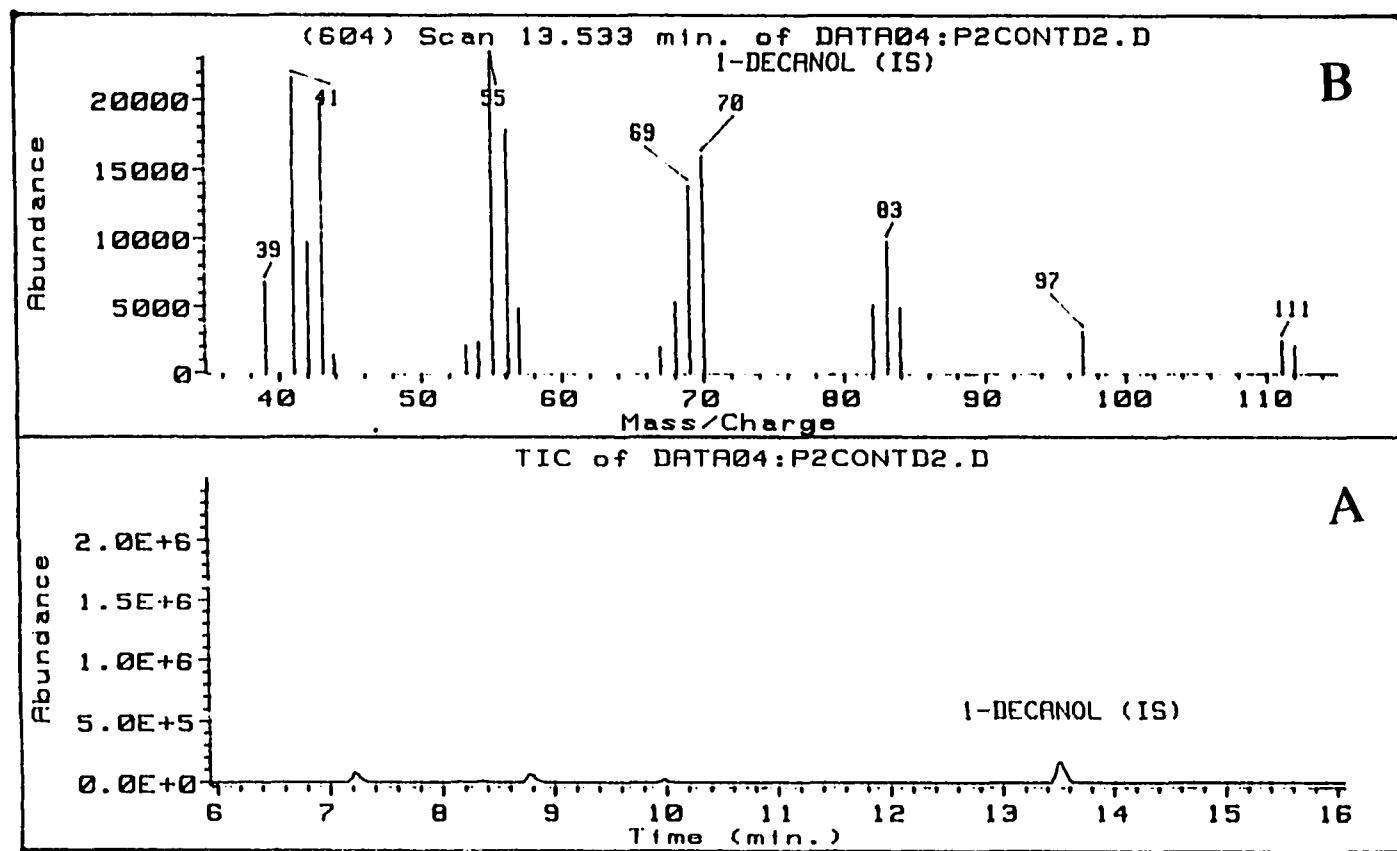


Fig. 9. Total ion chromatogram of extraction from 2 days *P. digitatum* culture without limonene addition (A). Mass spectrum at retention time 13.53 min (B).

Table 7.
Growth media evaluation for the growth of *P. digitatum*

Media	Composition (g/l)	Growth Evaluation*
MGP	Malt Extract (20), Glucose (20), Peptone (1)	+
Czapek's	NaNO ₃ (3), K ₂ HPO ₄ (1), MgSO ₄ .7H ₂ O (0.5), KCl (0.5), FeSO ₄ .7H ₂ O (0.01), Sucrose (30), Corn steep liquor (10)	+
YMG	Yeast extract (10), Malt extract (30), Glucose (10)	+
YMGP	Yeast extract (10), Malt extract (30), Glucose (10), Peptone (1)	++
PYP	Potato dextrase (24), Yeast extract (5), Peptone (5)	+++
MEA	Malt extract (20), Glucose (20), Peptone (1)	+
Abraham's	Glucose (10), Peptone (10), Malt extract (20), Yeast extract (3)	++++
Leonian's	Peptone (0.625), Maltose (6.25), malt extract (6.25), KH ₂ PO ₄ (1.25), MgSO ₄ .7H ₂ O (0.625), Yeast extract (1)	++
Hotop's	Glucose (30), Lactose (10), KH ₂ PO ₄ (0.5), (NH ₄) ₂ SO ₄ (5), Corn steep liquor (15)	+

*Growth evaluation scores were based on the growth appearance in flasks.

maximum α -terpineol production from limonene was found 1 day after the fungus was transferred from pre-culture inoculum (Fig. 10). The rate of α -terpineol production was $38.6 \pm 3.1 \mu\text{g/hr}$ ($926 \pm 74 \mu\text{g/ml}$) after 24 hr. incubation at 100 rpm and 28°C . A total of 11% of the added limonene (1%) in the reaction mixture was converted in 24 hrs.

Since growth medium was the same medium as used for pre-culture, almost no lag phase was seen. This bioconversion activity was only found during the early log phase of the growth cycle. The activity dropped dramatically after mid-log. The amount of α -terpineol production was a trace after 72 hrs. of growth (Fig. 10). This pattern was not affected by the age of the inoculum. One-day and 6-day old inocula were compared for the fungal growth and activity (Fig 11). In both cases, the limonene bioconversion was found in the early log phase of growth. The only difference between the cultures was the length of lag phase. In the 6-day old inoculum, the lag phase was 36 hrs longer than that from the 1-day old inoculum (Fig. 11). However, the pattern of growth and α -terpineol production of the two were the same.

2. Resting cells and α -terpineol production

Resting cells were tested for their ability to convert limonene to α -terpineol. Resting cells produced from two different growth stages were used. One was early log (6 hr. growth) and the other was mid-log cells (24 hr growth). The mycelia were resuspended into 50 mL of 0.05 M pH 7 citrate-phosphate buffer. Limonene (1%, v/v) was added and the flask was incubated at 100 rpm and 28°C . Five ml samples were taken every 12 hr for determination of α -terpineol concentrations. The specific activities, with substrate contact time, were plotted (Figure 12). α -Terpineol formation reached a

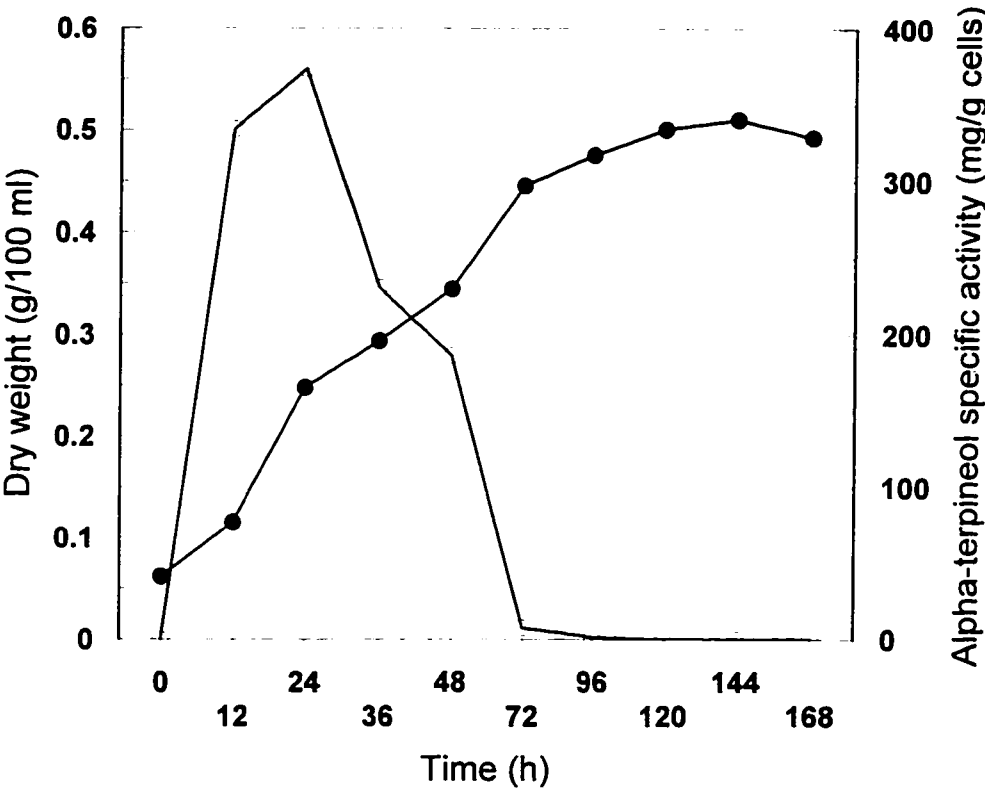


Fig. 10. Specific activity of bioconversion by *P. digitatum* growing cells at 28°C in 24 h.
Closed circle is mycelia dry weight and open circle is specific activity of α -terpineol

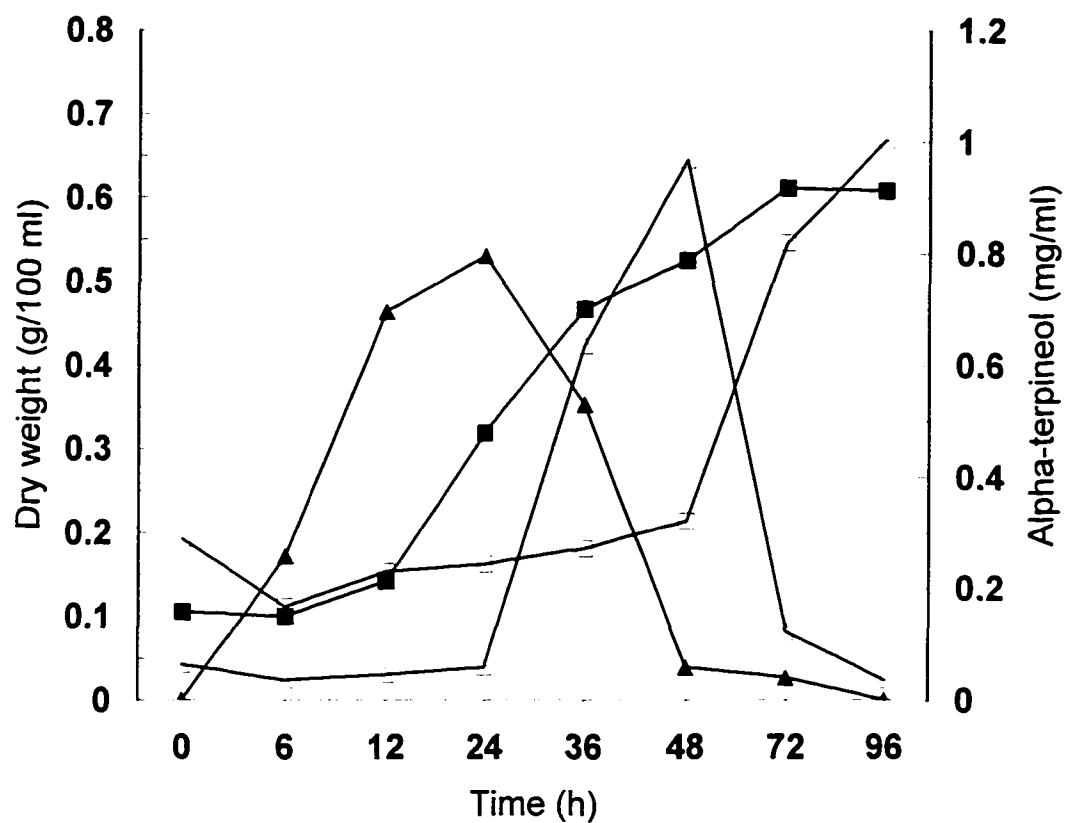


Fig. 11. Different age inocula, effect on growth and bioconversion activity of α -terpineol at 28°C. Assayed after 24 hrs. Closed and open squares are dry weights of mycelia from 1 day old inoculum and 6 day old inoculum respectively. Closed and open triangles are the α -terpineol production from 1 day old and 6 day old inoculum cultures.

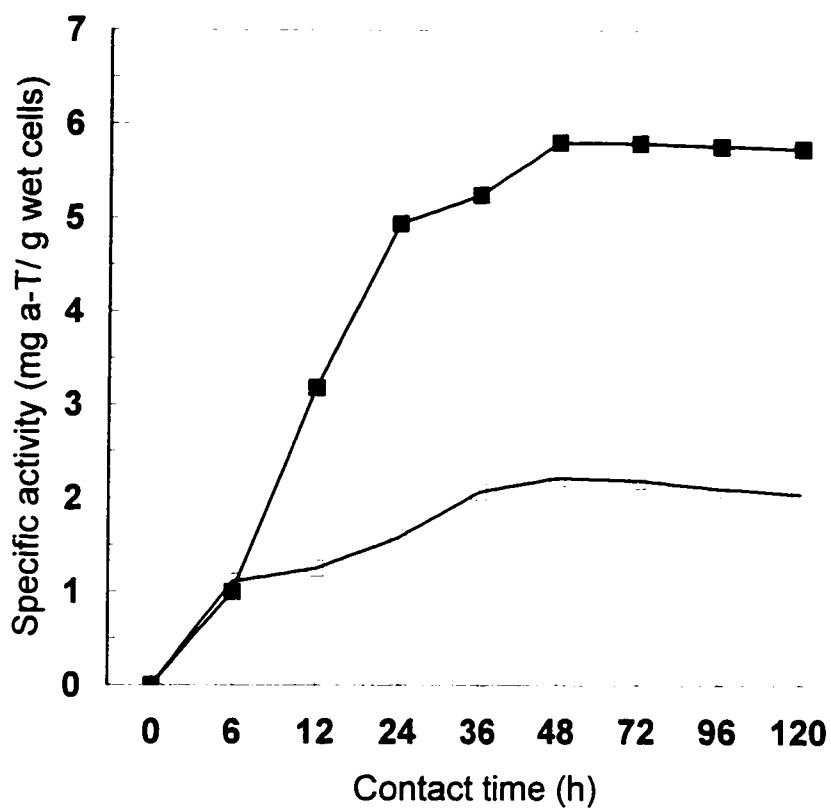


Fig. 12. Specific activity of *P. digitatum* resting cells for bioconversion of limonene. Open square is specific activity of 6 h growth resting cells. Closed square is specific activity of 24 h growth resting cells.

maximum and stopped after 48 hr in both cases. The higher specific activities were found with early-log phase resting cells. The maximum activity from these resting cells was about 5.74 mg α -terpineol per gram of wet cells per day. However, the highest specific activity from mid-log resting cells was only 2.16 mg/g wet cells per day. Its specific activity was almost three times less than that of resting cells from early-log phase.

3. Substrate induction

The bioconversion of limonene to α -terpineol was found to be catalyzed by an inducible enzyme system. Small amounts of limonene (200 ppm) were spiked into growing cell cultures at early-log, mid-log, stationary-phase, in each case 12 hrs prior to bioconversion assay. The bioconversion of induced cultures were compared with non-induced cultures which were used as controls. The bioconversion activities from three single inductions (Fig. 13), and the three continuous inductions (Fig. 14) were measured. Limonene induction increased α -terpineol production at all stages of fungal growth, the induction at all three stages showed a strong synergetic effect (Fig 13, 14). Production of α -terpineol over a 4-day period with induction at early-log, mid-log, stationary-phase, and all the three phases, increased bioconversion activity by 4, 4, 6, and 12 fold, respectively, compared with the control.

The bioconversion extent, the ratio of amount of product (α -terpineol) produced to the amount of substrate (limonene) added, was compared for induced early-log growing cells and non-induced cells over a range of substrate concentrations (Fig. 15). For induced cells, the conversion extent was greater than 50% when limonene concentration was less than 300 ppm. Over this concentration range, the lower the

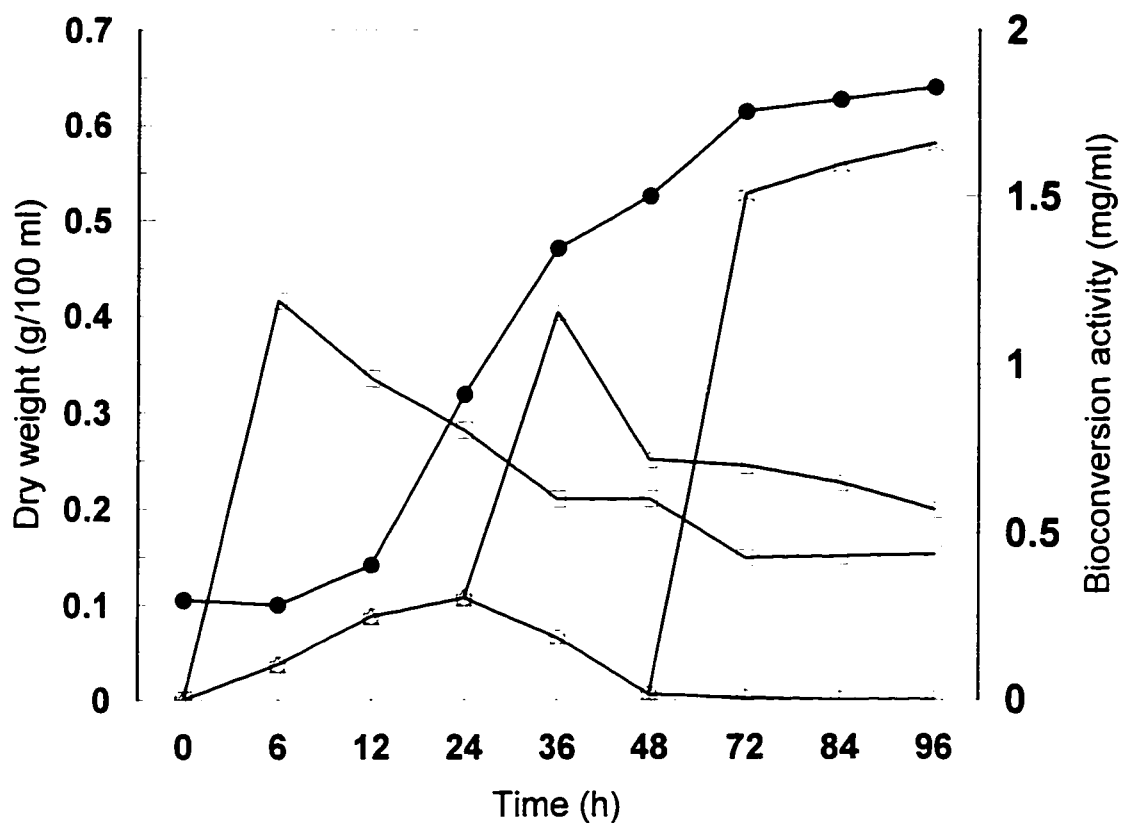


Fig. 13. Bioconversion after 200 ppm substrate induction at different growth stages. Activity was assayed at 28°C for 24 h. Closed circle is the dry weight of mycelia. α -Terpineol production after substrate induction at beginning growth (open square), mid-log (open triangle), stationary phase (open star), and the control without induction (open circle), is shown.

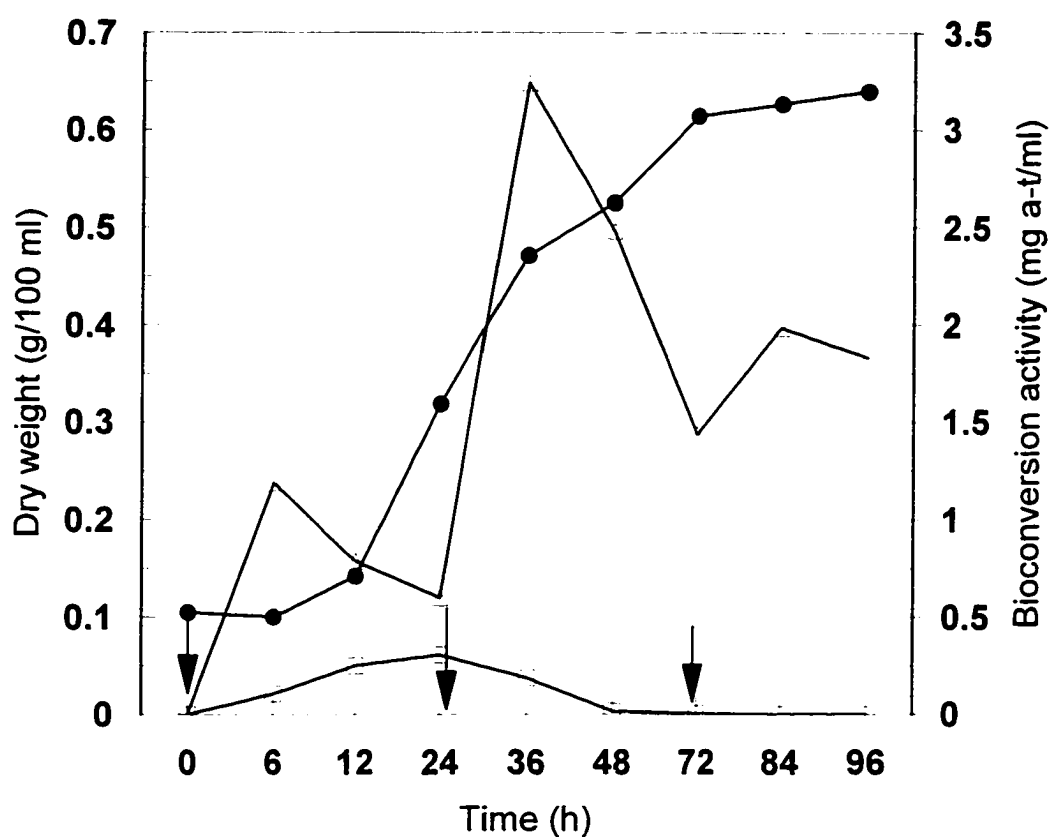


Fig. 14. Comparison of bioconversion activity with induction by 200 ppm substrate added at three points, arrows show the time of induction. Closed circle is the dry weight of fungal mycelia. Open square and triangle are α -terpineol production from non-induced control and the continuous induction respectively.

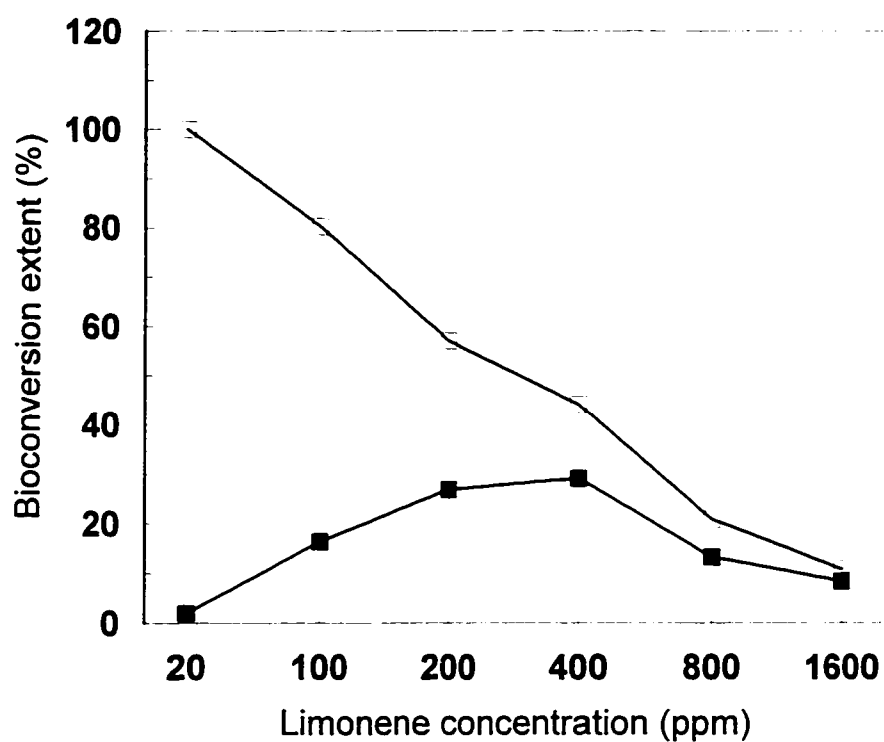


Fig. 15. Bioconversion extent comparison of induced growing mycelia from early-log phase and non-induced mycelia over a range of substrate concentrations. Open and closed squares are induced and non-induced mycelia respectively.

substrate concentration, the higher the bioconversion extent. Bioconversion extent from induced cells was greater than for non-induced cells in all concentration levels of the limonene used in the test (Fig., 15).

4. End product inhibition

As the concentration of α -terpineol accumulated in the reaction mixture, product inhibition was apparent (Fig. 16). The K_{iapp} of α -terpineol to this bioconversion was shown to be 12.08 mM. This means that the rate of product formation decreased by half when the inhibitor, the end product, α -terpineol, accumulated to about 12.08 mM (1.86 mg/ml) in the reaction mixture. From this plot, the V_{max} of the bioconversion (the reciprocal value of intercept point of Y axis) was about 4.9 mg/ml. This indicated that the maximum rate formation of α -terpineol was 4.9 mg/. The bioconversion stopped when the amount of α -terpineol reached this maximum because of end product inhibition.

5. Inhibitor studies

Many fungal hydroxylation reactions are catalyzed by a cytochrome P-450 monooxygenase system (Holland et al., 1987). This enzymatic system contains flavoprotein. Phenanthroline, an iron chelating agent, was found to inhibit the bioconversion (Fig. 17). Its inhibitory effect on free cells was greater than on immobilized fungal cells (Fig. 17). For free cells, the K_{iapp} of this inhibitor was 7.4×10^{-3} mM, while this value was 0.224 mM for immobilized cells. The difference in inhibitory effect from this inhibitor was 30 fold between free and immobilized cells.

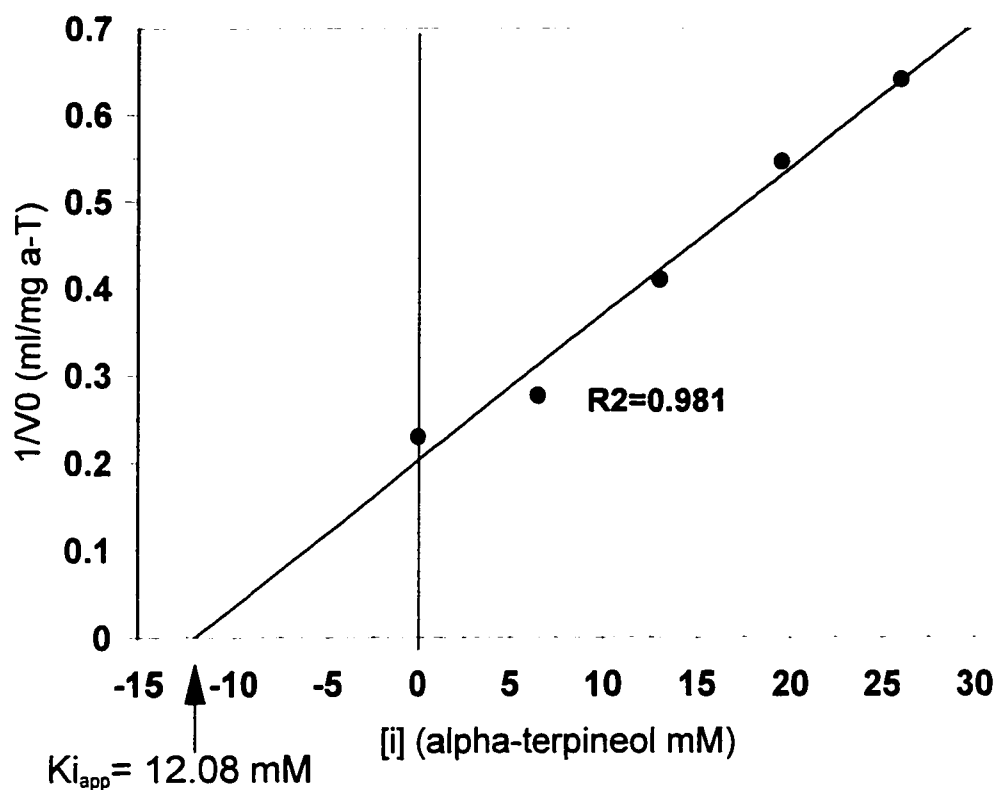


Fig. 16. Effect of α -terpineol concentration on free cell bioconversion of limonene by *P. digitatum*. Tests were conducted by addition of known amount of α -terpineol and monitoring its increase after adding limonene into 12 hr growing cells broth.

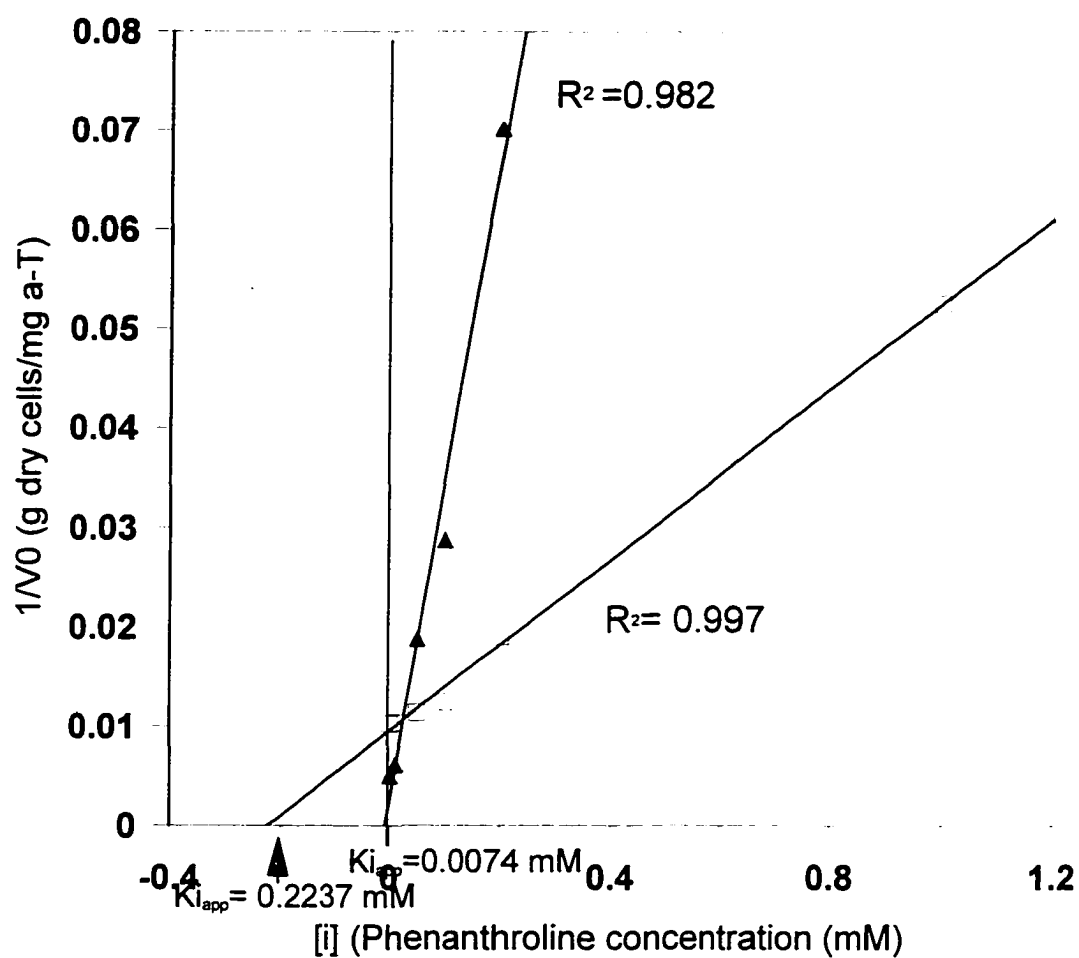


Fig. 17. Inhibition of phenanthroline on bioconversion of limonene by *P. digitatum* free and immobilized cells. Immobilized cells are shown by open square and free cells by closed triangle

6. K_m (app.) of bioconversion of limonene with free or immobilized cells

The Michaelis-Menten constant [K_m (app.)] of the bioconversion of limonene by *P. digitatum* was determined for both free cells and immobilized cells (Fig 18). A K_m (app.) of 29.0 μmol was obtained for this bioconversion when free cells were tested (Fig. 18). The maximal velocity [V_{max} (app.)] was 10.3 $\mu\text{mol}/\text{min}/\text{gram}$ of dry cells. When immobilized cells were used, k_m (app.) and V_{max} (app.) were 40.9 μmol and 9.5 $\mu\text{mol}/\text{min}/\text{gram}$ dry cells in beads respectively (Fig. 18). Immobilization did not change the V_{max} of this bioconversion. However, the K_m (app.) of this bioconversion decreased 75% with immobilized cells compared with free cells.

7. Stereochemical properties

In order to test stereochemical specificity of this bioconversion, (R)-(+)-, (S)-(-)-, and racemic limonene were tested as substrates. This fungus showed both stereospecificity and stereoselectivity in this bioconversion (Fig. 19). The fungus converted only R-(+)-limonene into (4R)-(+)- α -terpineol (Fig 19A), no (4S)-(-)- α -terpineol enantiomer was detected with a chiral GC. The fungus did not convert S-(-)-limonene (Fig. 19B). When racemic limonene was used only, the (R)-(+)-limonene was converted to (4R)-(+)- α -terpineol (Fig. 19C). The (S)-(-)-limonene used in this study was only of 96% optical purity so that a trace amount of α -terpineol was formed from the small amount of (R)-(+)-limonene present (Fig. 19B). (4R)-(+)- α -Terpineol, 1.301 mg, 0.103 mg and 0.709 mg was produced when (R)-(+)-, (S)-(-), and racemic form of limonene were used as substrates, respectively. The relative yields from (S)- (-)-, and

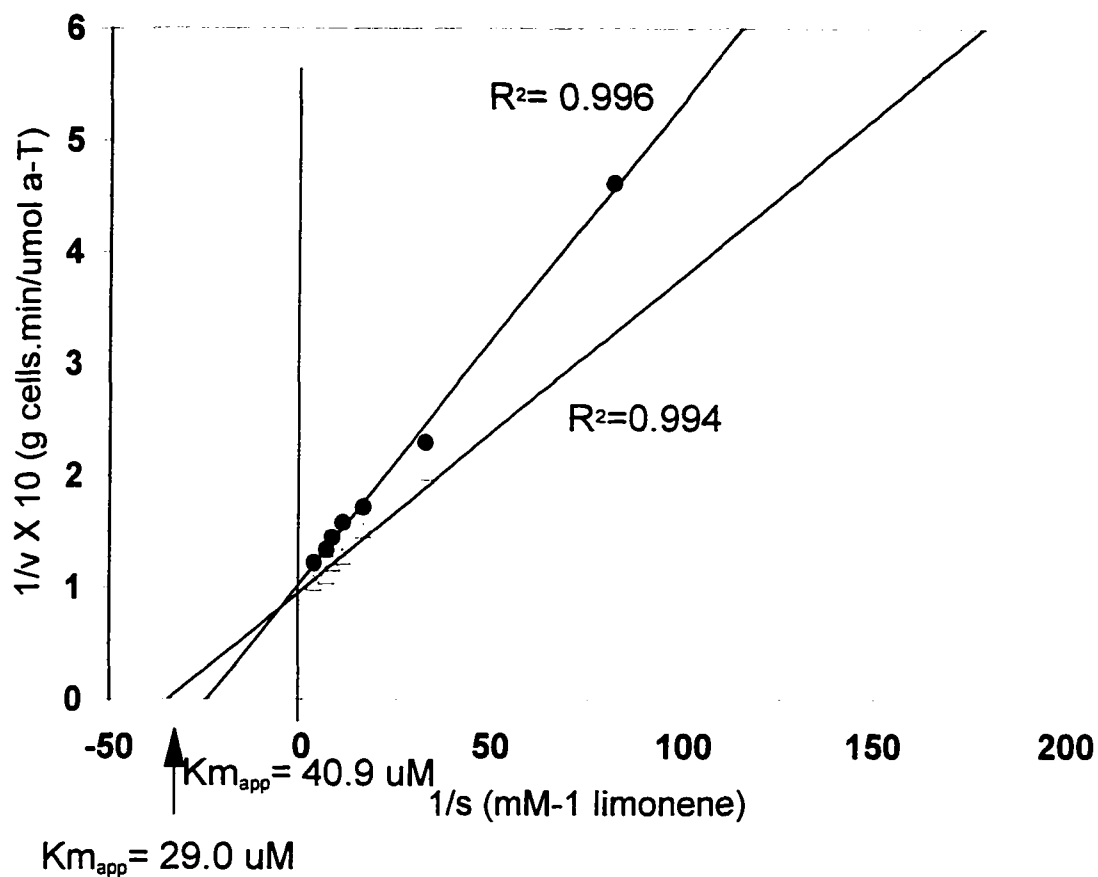


Fig. 18. Lineweaver-Burk plot showing the K_m and V_{max} for limonene bioconversion by growing and immobilized *P. digitatum* mycelia. Immobilized cells are shown by closed circle and free cells by open triangle.

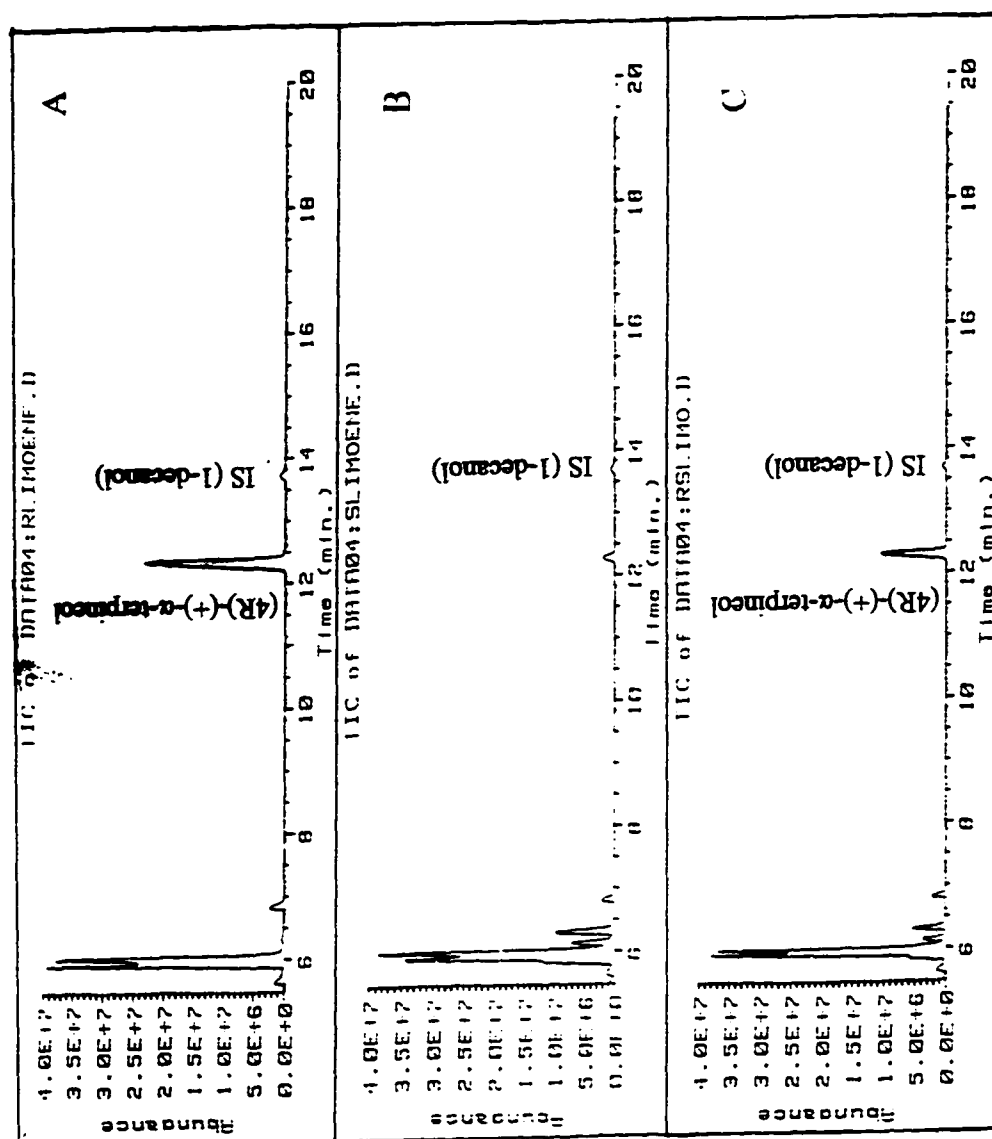


Fig. 19. Total ion chromatograms of bioconversion of (R)-(+)- (A), (S)-(-) (B), and racemic limonene (C) to α-terpineol by *P. digitatum*.

racemic limonene were 7.9% and 54% of that from (R)-(+)-limonene as the substrate, respectively.

III. Optimization of bioconversion conditions

1. Aeration

Oxygen was found to accelerate the bioconversion reaction. However, excess O₂ decreased bioconversion. Three different aeration rates were used (varying shaker speeds: 0, 100, and 200 rpm). The dissolved oxygen concentrations in these cultures were 0.77, 1.6, and 3.03 mg/l, respectively. Both free cells and immobilized cells were tested (Fig. 20). The highest bioconversion activity from free cells and immobilized beads was found at 1.6 mg/l of D.O (with 100 rpm shaking speed). At this aeration rate, free and immobilized cells produced 234 and 191 mg of α -terpineol per gram of dry cells in 24 hrs, respectively (Fig. 20). The activity from 3.03 mg/L D.O (200 rpm shake) was less than that from 1.6 mg/L D.O, 26 and 94 mg/g cells per day of α -terpineol for free and immobilized cells, respectively. The lowest activity was from the standing cultures, with 0.77 mg/L of D.O..

Time courses for bioconversion activity for immobilized cells and free cells under different aeration rates were constructed (Fig. 21). Under aeration rates with both 100 rpm and standing culture, a higher bioconversion with time was obtained from free cells rather than immobilized cells. However, bioconversion was higher with immobilized cells than with free cells when 200 rpm of aeration rate was used (Fig 21). Under this aeration, activities of both free and immobilized cells reached the maximum level within

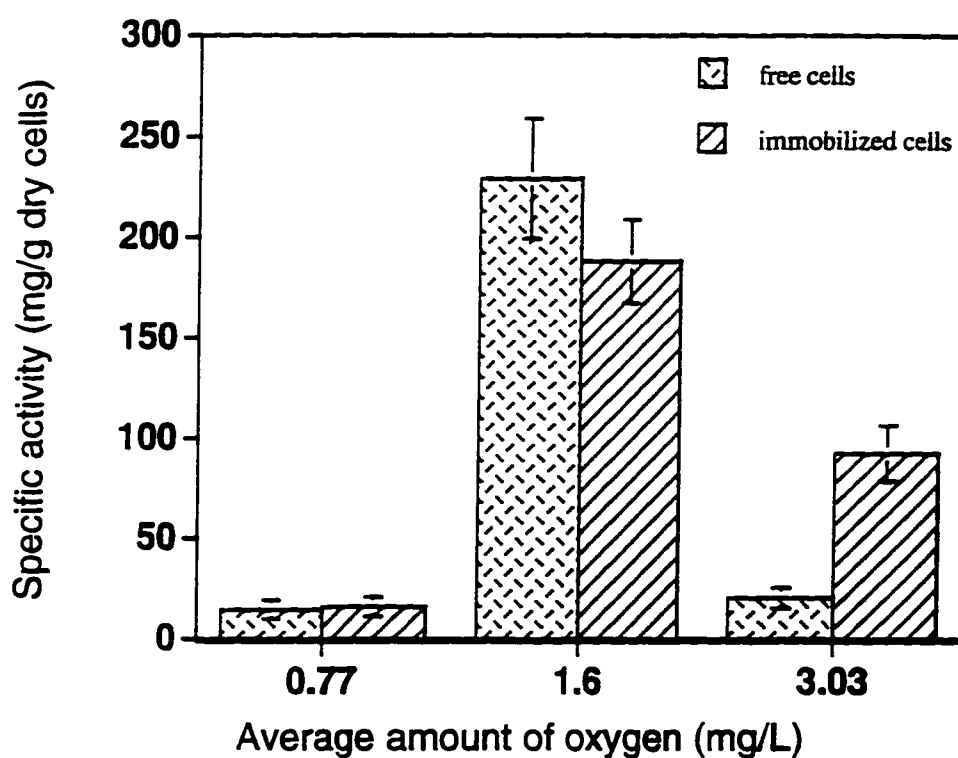


Fig. 20. Oxygen effect on specific activity of free and immobilized cells. Free cells were 36 h growing cells. Activity was measured at 28°C for 24 h. Value are given as mg/g dry cell weight \pm SD.

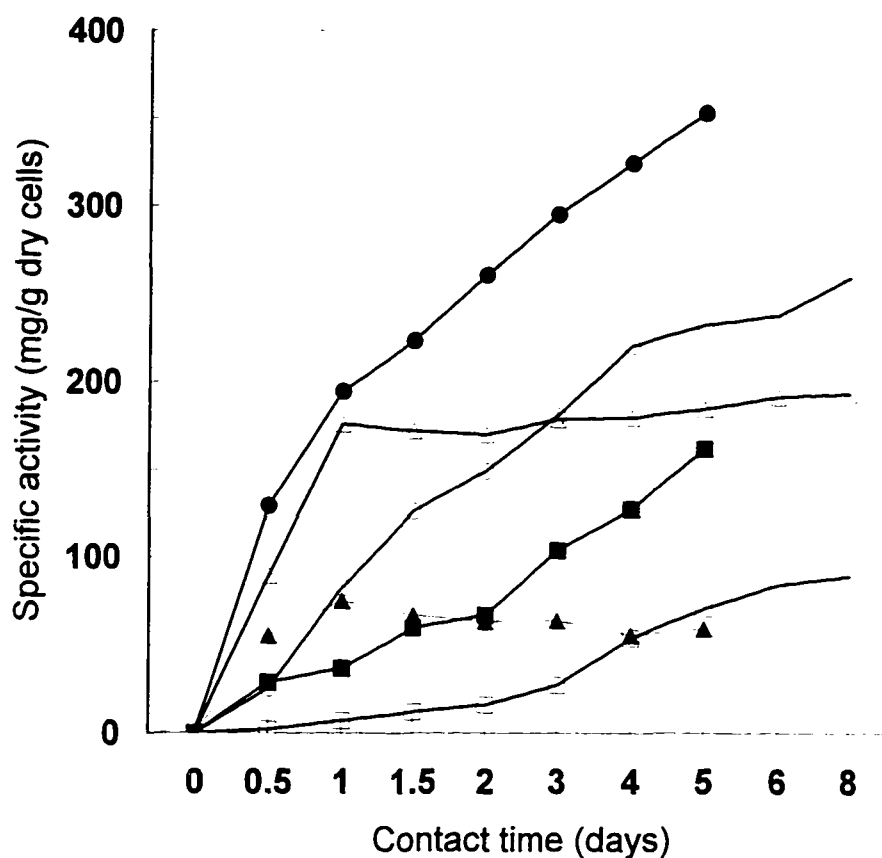


Fig. 21. Time courses for bioconversion activity of free and immobilized *P. digitatum* cells assayed under different aeration rates. Free cells were 12 h growing cells. Activity was assayed at 28°C for 24 hr. Closed squares, circles, and triangles indicate free cells assayed with standing culture, 100 rpm, and 200 rpm culture respectively. Opened squares, circles, and triangles show immobilized cells with standing culture, 100 rpm, and 200 rpm respectively.

1 day of substrate contact (Fig 21). The bioconversion then stopped and the amount of α -terpineol remained level for the rest of time. This pattern was different from those of 100 rpm and standing culture. In these two cases, the activity of bioconversion increased slowly for 5 days (Fig 21).

2. Temperature

Optimum temperature for bioconversion for both free and immobilized cells was determined. No difference was found in the optimum temperature between the two forms, but immobilized cells had a wider temperature activity range (Fig. 22). Over a range from 28 to 36 °C, immobilized cells showed maximum α -terpineol yields. Free cells only achieved the maximum yield over a temperature range from 28 to 32°C. All further experiments were conducted at 28°C.

3. pH

The pH effect on bioconversion was investigated using free, resting cells and immobilized cells. High bioconversion activity was found across a wide pH range, from 3.5 to 8, whether free resting cells or immobilized cells were used. Four distinct peaks were identified in the pH profiles from both free resting cells and immobilized cells (Fig. 23). Highest bioconversion was found at pH 4.5 for free resting cells, while the highest activity for immobilized cells was obtained at pH 7.

4. Substrate concentration

Generally, organic solvents are toxic to cells, especially at high concentrations (Lanne et al., 1987a). To determine the optimum concentration of limonene for bioconversion to α -terpineol, growing cells and immobilized cells were tested over a range of 0.02 to 8 % (v/v). For growing cells, the optimum substrate concentration was

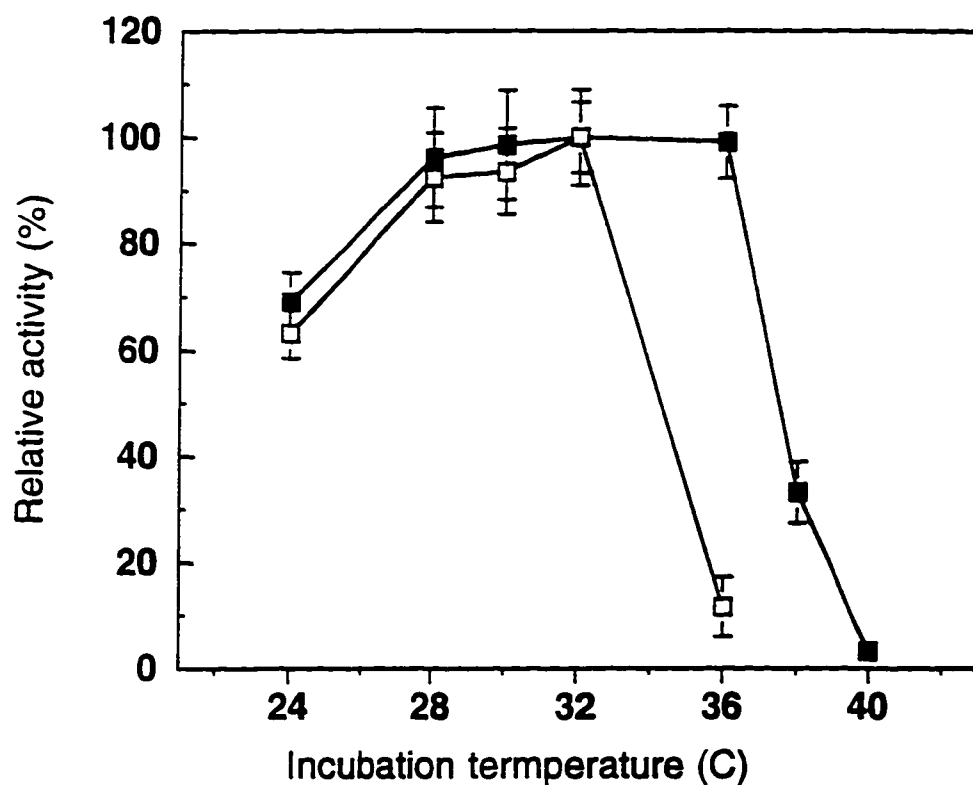


Fig. 22. Temperature optima for bioconversion by *P. digitatum* with free and immobilized cells. Free cells were 5 ml of 36 h old cells. Three grams of immobilized cells were used. All assays were conducted by comparison with assay at 28°C. Closed and open squares indicate free and immobilized cells respectively. Error bars show \pm SD. Values are mean values of 3 trials.

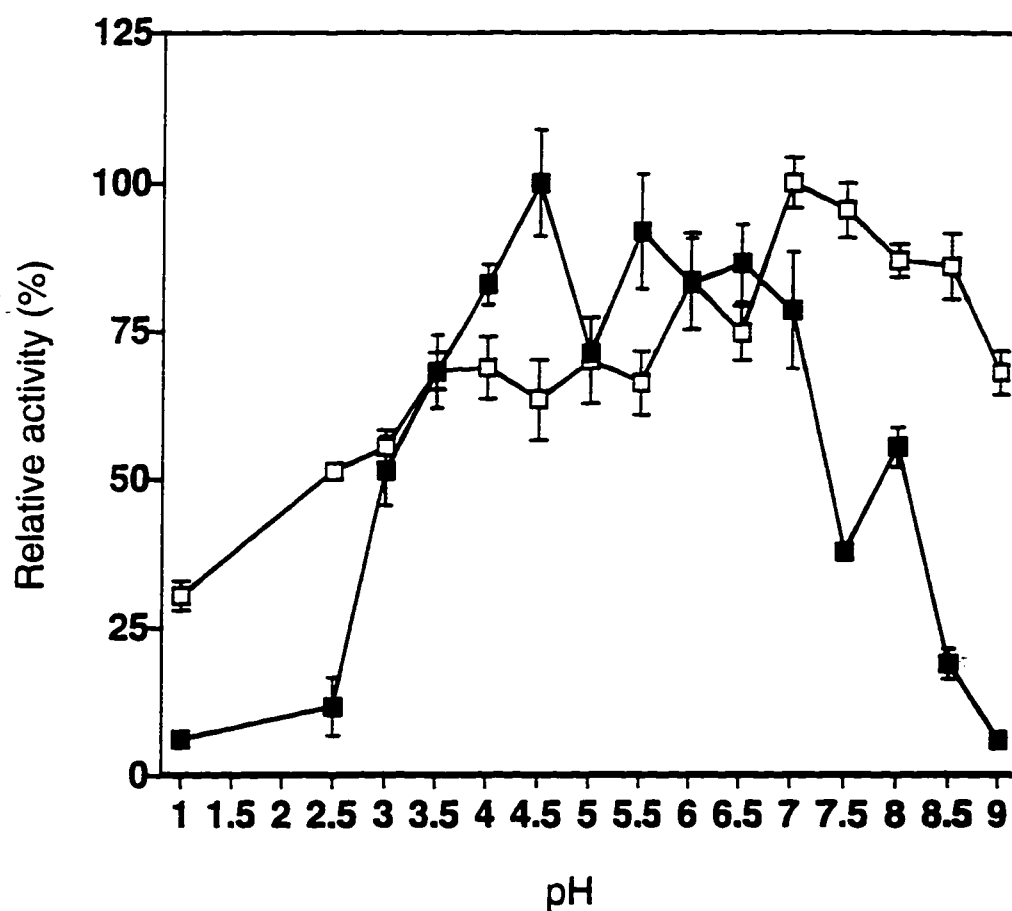


Fig. 23. pH Optima for bioconversion of limonene by *P. digitatum* with free and immobilized cells. Citrate phosphate buffer (0.05 M) was used for pH ranges from 2,5 to 8, 0.1 M of glycine NaOH buffer for pH 8.5 and 9.0. pH 1 was 0.1 M of HCl. Free cells were 12 h growth resting cells (0.17 g). Three grams of immobilized cells were used. All assays were conducted at 28°C for 24 h. Open and closed squares are pH profiles of immobilized and free cells respectively. Error bars show \pm SD. Values are mean values of 3 trials.

about 1% (v/v) (Fig. 24). The bioconversion extent of limonene, a measurement of bioconversion efficiency, decreased as the concentration of substrate increased. Increases in substrate concentrations above 1% (v/v) did not bring about a further increase in the amount of α -terpineol produced. On the other hand, substrate concentrations from 1% to 8% (v/v) did not significantly affect the activity of this bioconversion with free cells. Apparently, there was no substrate toxicity up to 8% substrate concentration.

For immobilized cells, the optimum substrate concentration was higher because of alginate gel protection of fungal cells from bulk contact with the substrate (Fig 24). The amount of α -terpineol increased with substrate concentration up to 8% limonene. No difference in product yield was detected between substrate concentrations of 4% and 8%. A 4% substrate level was the optimum substrate concentration for immobilized cells. The effect of substrate concentration on bioconversion extent for immobilized cells was the same as for free cells (Fig. 24). The percentage bioconversion extent decreased with an increase in substrate concentration. The maximum of bioconversion extent for immobilized cells was only about 50%, whereas it was as high as 100% for free cells at low substrate concentration.

5. Reaction time

The optimum substrate contact time for both growing and resting cells for maximum α -terpineol production was determined. Resting cells were obtained from 6-hr old broth cultures. The optimum substrate contact time with limonene for both growing and resting cells was between 24 and 48 hrs (Fig. 25). For immobilized cells, the optimum contact time was slightly longer than for free cells (Fig. 25). The optimum

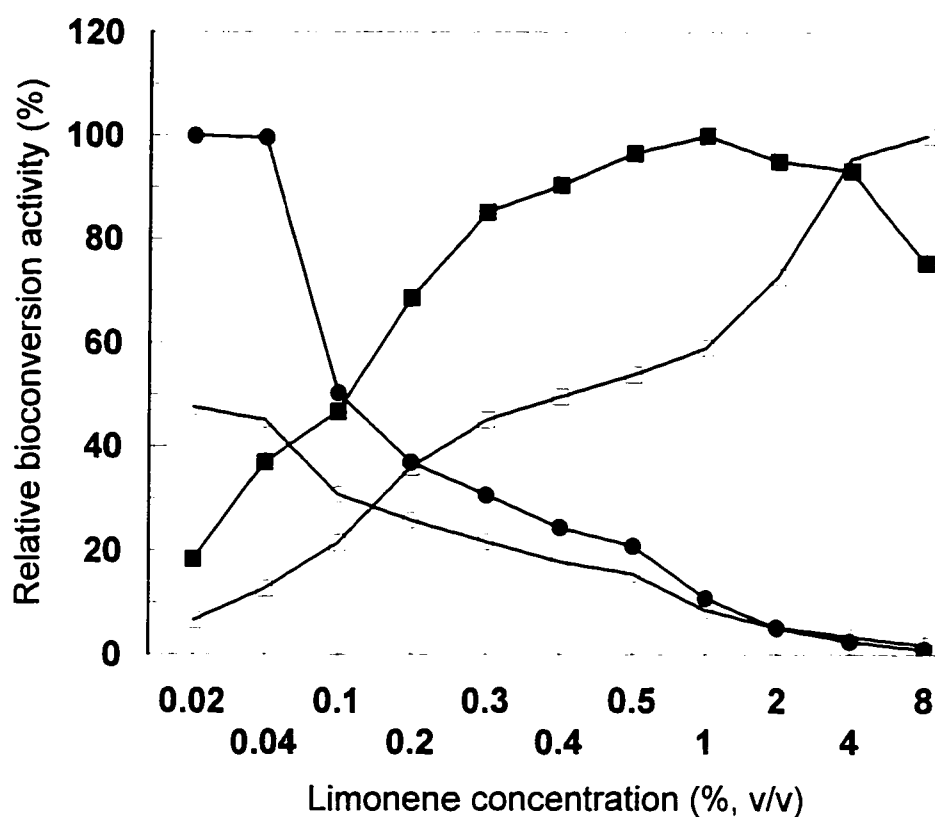


Fig. 24. Effect of substrate concentration on bioconversion activity for free and immobilized *P. digitatum* mycelia. Free cells were 5 ml of 36 hr growing cells. Three grams of immobilized cells were used. Each assay was conducted at 28°C for 24 hrs. Closed squares and circles show relative activity and bioconversion extent for free cells. Opened squares and circles indicate relative activity and bioconversion extent for immobilized cells.

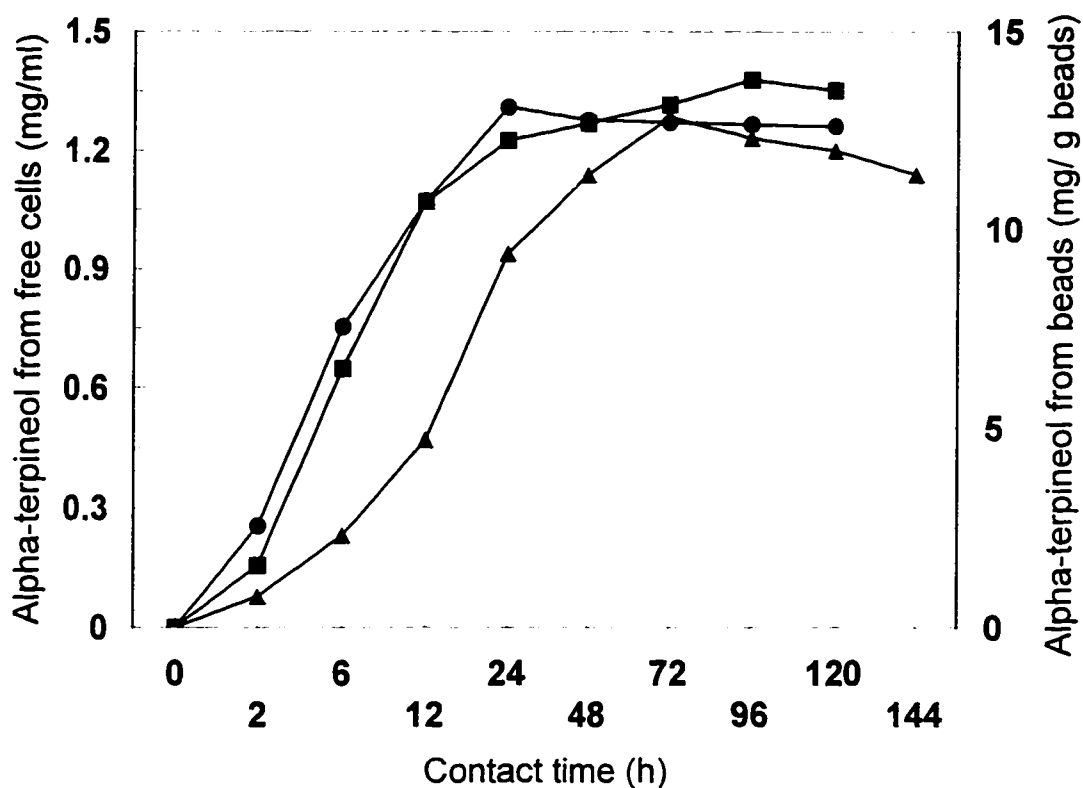


Fig. 25. α -Terpineol production as a function of substrate contact time for growing cells, resting cells, and immobilized cells. Resting cells used were 0.17 g of 6 hr growing mycelia in 5 ml of buffer (pH 4.5). Three grams of immobilized cells in 5 ml buffer (pH 7) were used for each assay. All assays were conducted at 28°C for 24 hrs. Closed circles and squares are growing cells and resting cells respectively. Close triangles show immobilized cells.

substrate contact time for immobilized cells was between 3 and 4 days. After this period, no more α -terpineol was produced (Fig. 25). Again, the highest bioconversion extent with immobilized cells was 45.81% (less than 50%) when a 1% substrate concentration was used.

IV. Bioconversion of limonene with immobilized fungal cells

1. Stability of immobilized beads

The storage stability of immobilized beads, at -20°C and 4°C, based on activity was tested (Fig. 26). The activity of bioconversion of immobilized cells stored at -20°C dropped significantly with time. The activity drop was almost a negative straight line with slope of -12.25 % activity loss per day compared with the activity on the first day of the storage. After 10 days storage, the activity was totally lost. In contrast, the activity increased slightly in beads stored at 4°C. The activity of the beads after 14 days was $137 \pm 20\%$ of its activity when they were freshly made.

2. Batch and repeated batch bioconversions

Bioconversion with immobilized beads in batch form was tested. The conditions chosen were the optimum conditions previously tested except that limonene concentration was 1% (v/v). The aeration was 1.6 mg/l D.O (with 100 rpm shaking), temperature of 28°C, pH 7.0, 1% (v/v) Tween 80. The fungi were immobilized in early log phase. The product concentration was monitored daily after the substrate was added. The highest α -terpineol specific activity (12.83 mg/ g beads d) was obtained 3 days after limonene addition. The bioconversion extent was 45.81% (Table 8).

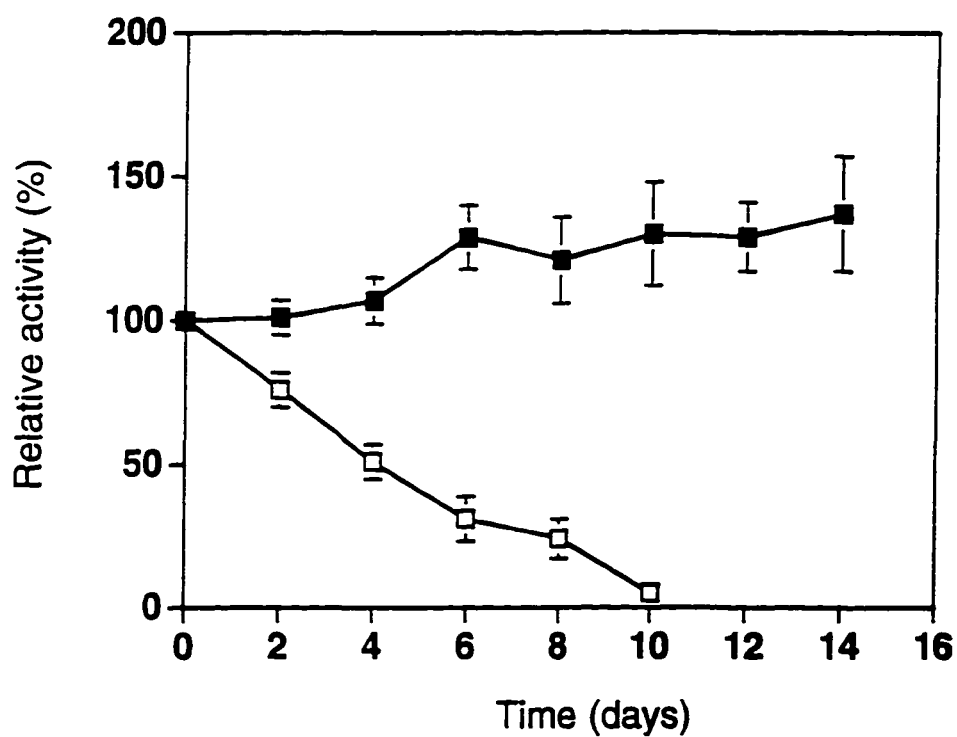


Fig. 26. Storage stability of immobilized beads for -20°C and 4°C based on activity. Closed and open square indicates relative activity under 4°C and -20°C storage respectively. Values are an average of 3 trials. Error bars show the SD.

Table 8.
Bioconversion activity of batch and repeated batch form

cycle	runing time (days)	specific activity (mg/g beads d)	α -terpineol productivity (mg/d)	bioconversion extent (%)
1	0	0	0	0
	1	9.39	281.7	33.53
	2	11.39	341.7	40.67
	3	12.83	384.9	45.81
	4	12.31	369.3	43.97
	5	11.99	359.7	42.81
	6	11.39	341.7	40.68
2	0	0	0	0
	1	1.23	36.9	4.39
	2	2.83	84.9	10.10
	3	4.09	122.7	14.59
	4	4.24	127.2	15.16
	5	3.91	117.3	13.95
	6	3.68	110.4	13.14
	7	3.66	109.8	13.06
	8	3.50	105.0	12.50
3	0	0	0	0
	1	0.61	18.3	2.19
	2	0.65	19.5	2.33
	3	0.66	19.8	2.35
	4	0.58	17.4	2.07
Regeneration of the beads with growth medium for 3 days				
4	0	0	0	0
	1	0.57	17.1	2.02
	2	1.18	35.4	4.22
	3	2.25	67.5	8.04
	4	2.92	87.6	10.44
	5	2.92	87.6	10.41
5	0	0	0	0
	1	2.43	72.9	8.67
	2	2.80	84.0	10.00
	3	3.18	95.4	11.37
	4	3.20	96.0	11.43

Repeated-batch experiments were carried out to investigate the possibility of reusing immobilized cells for this bioconversion (Table 8). The results indicated that the specific productivity of α -terpineol dropped from 346.5 mg/d to 101.8 mg/d from the first batch to the second batch. The productivity from the second batch was only about one third of that from the first batch (Fig. 27). The productivity dropped even more dramatically after the second batch. The average of productivity of the third batch was only 15% of that from the second batch and 6% of the first batch. According to some studies (Schlosser and Schmauder, 1991; Vidyarthi and Nagar, 1994), the activity of immobilized cells can be improved by a regeneration process after several uses. Regeneration was conducted after the third batch. The immobilized cells were regenerated by exposure to the growth medium for three days. Then, the batch bioconversions were repeated. The 4th and 5th batch bioconversions were tested. Comparing the beads after regeneration (the 4th batch) with the batch before (3rd batch) shows the average productivity of α -terpineol was increased 3 fold from 18.77 mg/d to 59.02 mg/d (Fig. 27). In the 5th batch, the productivity from the 30 gram alginate beads was still maintained at 87.08 mg/d average. This indicated that a regeneration process after two or three runs will help to increase the productivity.

3. Continuous bioreactor

Continuous bioconversion of limonene to α -terpineol was tested with immobilized fungal beads in a bioreactor. Since aeration significantly altered bioconversion activity, α -terpineol production with immobilized cells in the bioreactor was tested with 4 different aeration rates (0, 0.2, 0.3, and 0.4 SLPM). Each aeration rate was tested against two flow rates (0.0072 and 0.0144 h⁻¹ dilution). In order to compare

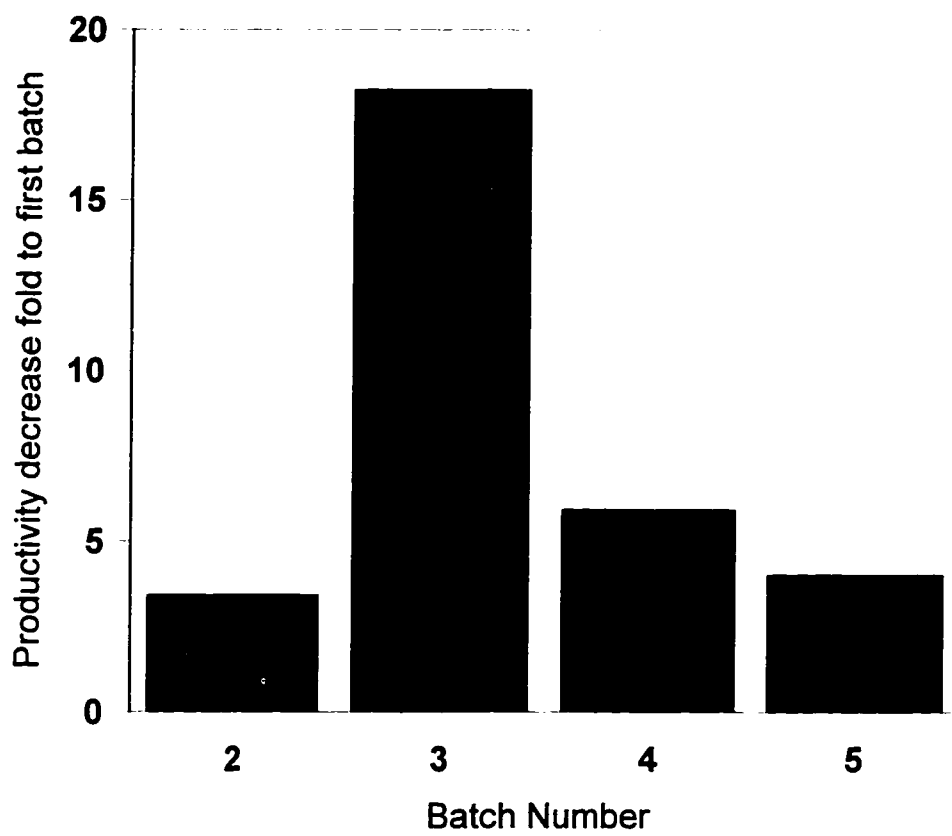


Fig. 27. Productivity comparison of repeated batch to first batch with immobilized beads. Beads were regenerated after the third batch.

productivities between each run, they were calculated based on the productivity relative to a flask control containing 30 grams of the same type of beads used in the bioreactor. Figures 28 and 29 show the bioconversion activities relative to the controls with time courses at two flow rates and 4 different aeration rates. The average α -terpineol productivity with time is shown in Figure 30.

At a fixed (0.0072 h^{-1}) dilution rate, the highest α -terpineol productivity was obtained with an aeration rate of 0.3 SLPM. Productivity was 2.6 fold higher than the control (Fig 30). The second highest productivity was obtained with 0.2 SLPM aeration, 1.2 fold that of the control. The lowest productivity was obtained from the 0 SLPM with only average productivity of 45.8% of that of the control (Fig. 30). The longest half-life of the column was about 20 days at an aeration of 0.2 SLPM. The half-life of the bioconversion column at 0.3 SLPM was about 6 days. The half-life of the column with 0.4 SLPM of aeration was the same as 0.3 SLPM (Fig 28).

At 0.0144 h^{-1} dilution rate and 0.3 SLPM aeration, the productivity was almost 4 times that of the control. The half-life of the column at this aeration rate was 6 days (Fig 29), which did not change with an increase in dilution rate from 0.0072 to 0.0144 h^{-1} .

V. Bioconversion of limonene in organic solvent systems

The effects of 22 different solvents or surfactants as co-solvents were tested for their ability to enhance this bioconversion, with both free and immobilized cells. The results were listed in Tables 9 and 10. The effect of each solvent on the bioconversion was different for free or immobilized cells. In case of free cells, dioctylphthalate (1.5%) significantly increased this bioconversion (Table 10). Compared with the control (no co-

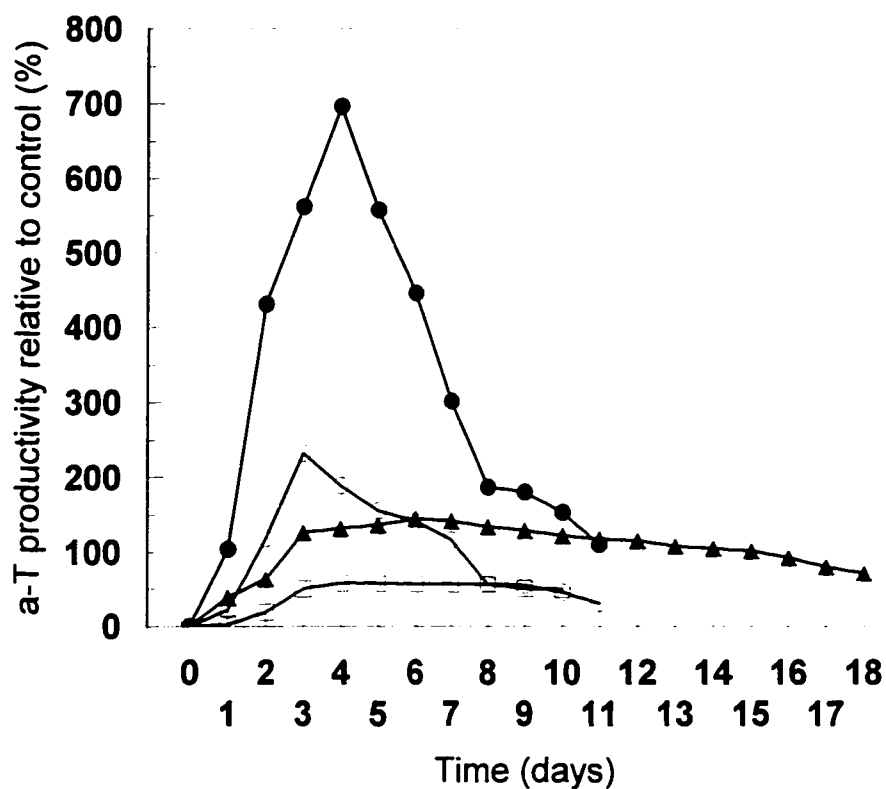


Fig. 28. Comparison of different aeration rates on α -terpineol productivity relative to control of immobilized cells with flow rate of 0.0072 h^{-1} in an air-lift bioreactor. One hundred and fifty grams of immobilized beads were used in each run with an air-lift bioreactor. Each control contained 30 g of the same beads in a 250 ml flask. All runs were conducted under room temperature. Open square, closed triangle, closed circle, and open circle indicates aeration rates with 0, 0.2, 0.3 and 0.4 slpm respectively.

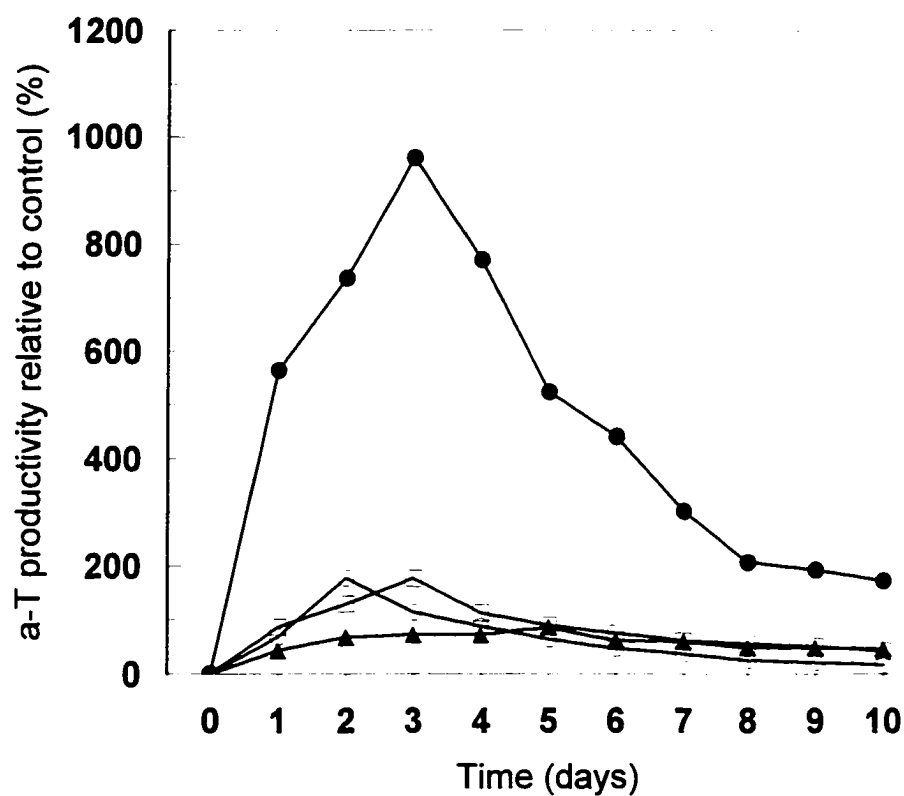


Fig. 29. Comparison of different aeration rates on α -terpineol productivity relative to control of immobilized cells with flow rate of 0.0144 h^{-1} in an air-lift bioreactor. Open square, closed triangle, closed circle, and open circle indicate aeration rates with 0, 0.2, 0.3 and 0.4 slpm respectively.

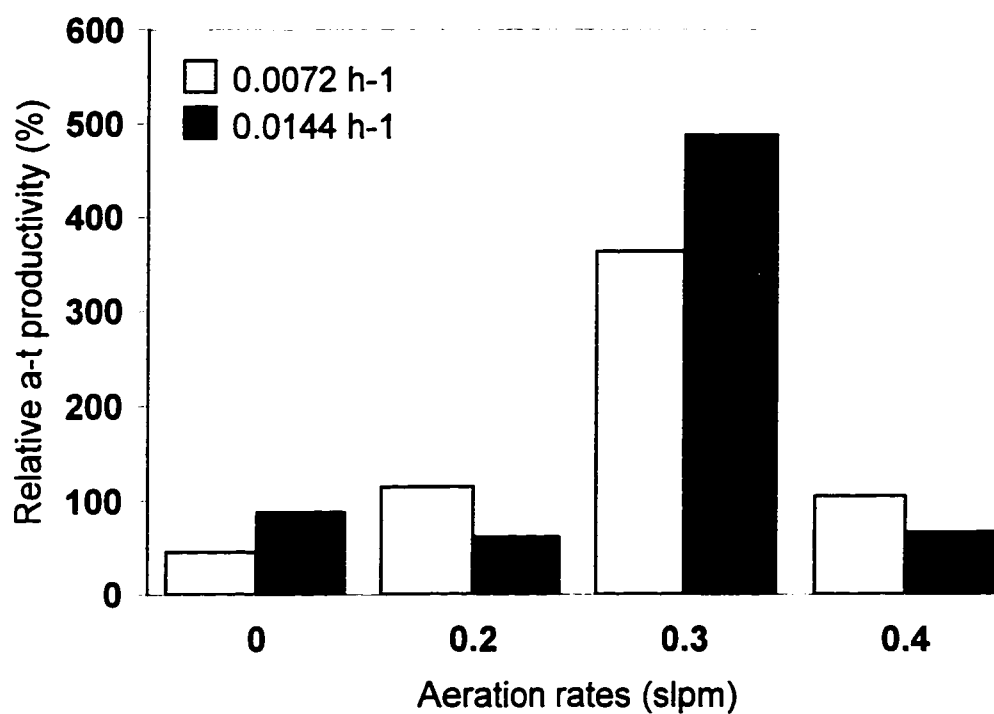


Fig. 30. Relative α -terpineol productivity with time at different aeration rates with immobilized cells in a continuous bioreactor.

Table 9.
Effect of addition of organic solvents and surfactants as co-solvents of substrate on immobilized beads for the bioconversion of limonene to α -terpineol.

Solvent and concentration (%, v/v)	Log P value#	α -Terpineol (mg/g beads) \pm SD	Relative activity (%) \pm SD
control (no solvent)	NA	0.69 \pm 0.041	100 \pm 6%
Tween 80 (0.1%)	-	1.508 \pm 0.136	218 \pm 9%
Triton 100-X (0.1%)	-	1.381 \pm 0.221	200 \pm 16%
polyethylene glycol (2%)	-	1.073 \pm 0.054	155 \pm 5%
propylene glycol (2%)	-1.4 *	0.908 \pm 0.055	131 \pm 6%
dipropylene glycol (2%)	-1.4*	0.911 \pm 0.064	132 \pm 7%
tripropylene glycol (2%)	-1.5*	0	0
glycerol (2%)	-3.0*	0.868 \pm 0.133	126 \pm 13%
dimethylphthalate (1.5%)	2.3	0.822 \pm 0.123	119 \pm 15%
diethylphthalate (1.5%)	3.3	0.246 \pm 0.029	36 \pm 12%
dioctylphthalate (1.5%)	9.6	0.636 \pm 0.108	92 \pm 17%
ethyl decanoate (1.5%)	4.9	0.618 \pm 0.117	90 \pm 19%
methanol (1.5%)	-0.76	0.662 \pm 0.046	96 \pm 7%
o-xylene (1.5%)	3.1	0.107 \pm 0.004	15 \pm 4%
cyclohexane (1.5%)	3.2	0.315 \pm 0.022	46 \pm 7%
p-cymen (1.5%)	4.1	0.408 \pm 0.049	59 \pm 12%
butylbenzoate (1.5%)	3.7	0.329 \pm 0.053	48 \pm 16%
tetradecane (1.5%)	7.6	0.399 \pm 0.052	58 \pm 13%
ethanol (2%)	-0.24	0.572 \pm 0.046	83 \pm 8%
n-amyl alcohol (2%)	1.3	0.012 \pm 0.000	2 \pm 2%
hexane (2%)	3.5	0.312 \pm 0.028	45 \pm 9%
isooctane (2%)	4.5*	0.446 \pm 0.094	65 \pm 21%
dimethyl-sulfoxide (2%)	-1.3	0.741 \pm 0.044	107 \pm 6%

Values following Lanne et al., 1987.

* Values were calculated based on Lanne et al., 1987.

Table 10.
Effect of addition of organic solvents and surfactants as co-solvents of substrate
on growing cells for the bioconversion of limonene to α -terpineol.

Solvent and concentration (%, v/v)	Log P Value#	α -Terpineol (mg/g beads) \pm SD	Relative activity (%) \pm SD
control (no solvent)	NA	0.532 \pm 0.043	100 \pm 8%
Tween 80 (0.1%)	-	0.500 \pm 0.075	94 \pm 15%
Triton 100-X (0.1%)	-	0.415 \pm 0.091	78 \pm 22%
polyethylene glycol (2%)	-	0.652 \pm 0.046	123 \pm 7%
propylene glycol (2%)	-1.4 *	0.548 \pm 0.055	103 \pm 10%
dipropylene glycol (2%)	-1.4*	0.566 \pm 0.051	106 \pm 9%
tripropylene glycol (2%)	-1.5*	0	0
glycerol (2%)	-3.0*	0.580 \pm 0.093	109 \pm 16%
dimethylphthalate (1.5%)	2.3	0.234 \pm 0.042	44 \pm 18%
diethylphthalate (1.5%)	3.3	0.093 \pm 0.010	17 \pm 11%
dioctylphthalate (1.5%)	9.6	1.464 \pm 0.410	275 \pm 28%
ethyl decanoate (1.5%)	4.9	1.173 \pm 0.270	220 \pm 23%
methanol (1.5%)	-0.76	0.614 \pm 0.049	115 \pm 8%
o-xylene (1.5%)	3.1	0	0
cyclohexane (1.5%)	3.2	0	0
p-cymen (1.5%)	4.1	0.045 \pm 0.002	9 \pm 4%
butylbenzoate (1.5%)	3.7	0.060 \pm 0.004	11 \pm 6%
tetradecane (1.5%)	7.6	0.180 \pm 0.014	34 \pm 8%
ethanol (2%)	-0.24	0.245 \pm 0.027	46 \pm 11%
n-amyl alcohol (2%)	1.3	0	0
hexane (2%)	3.5	0.144 \pm 0.007	27 \pm 5%
isooctane (2%)	4.5*	0.293 \pm 0.041	55 \pm 14%
dimethyl-sulfoxide (2%)	-1.3	0.591 \pm 0.053	111 \pm 9%

Values following Lanne et al., 1987.

* Values were calculated based on Lanne et al., 1987

solvent addition), α -terpineol yield was increased by 2.5 fold. Ethyl decanoate increased the product yield by 2.2 fold. Methanol, often used to improve the reaction rate of the bioconversion of steroids (Sode et al., 1989), showed a small positive effect on this bioconversion. It increased the yield by 15% at a concentration of 1.5% (v/v). Other alcohols, such ethanol and n-amyl alcohol, were strongly inhibitory, particularly n-amyl alcohol which completely stopped all bioconversion.

Dioctylphthalate and methanol were tested with free cells to optimize the bioconversion. For methanol, the co-solvent concentrations ranged from 0 (control) to 8% (v/v) (Fig. 31). The highest product yield was obtained at a methanol concentration of 0.5% (v/v). At this concentration, the yield increased by 45%. The bioconversion extent went from 27.3% to 39.5%. Dioctylphthalate was tested over a 0 to 4% (v/v) range (Fig. 32). A 2% (v/v) dioctylphthalate concentration in the mixture produced the highest α -terpineol yields. At this concentration, the product yield increased about 2.5 fold. The bioconversion extent increased from 35.6% (control) to 92.4% (2% co-solvent). When dioctylphthalate concentration was above 2%, the yield of the product decreased. However, the toxicity of this organic solvent was not obvious until the concentration was greater than 4% (v/v) (Fig 32).

Organic co-solvents for immobilized cells effects were different from those of free cells (Table 9). Dioctylphthalate (1.5%, v/v) and ethyl decanoate(1.5%, v/v) had no significant effect on bioconversion with immobilized cells. The relative activities for these two solvents compared with the control (no solvent additions) were $92\pm 17\%$, and $90\pm 19\%$, respectively.

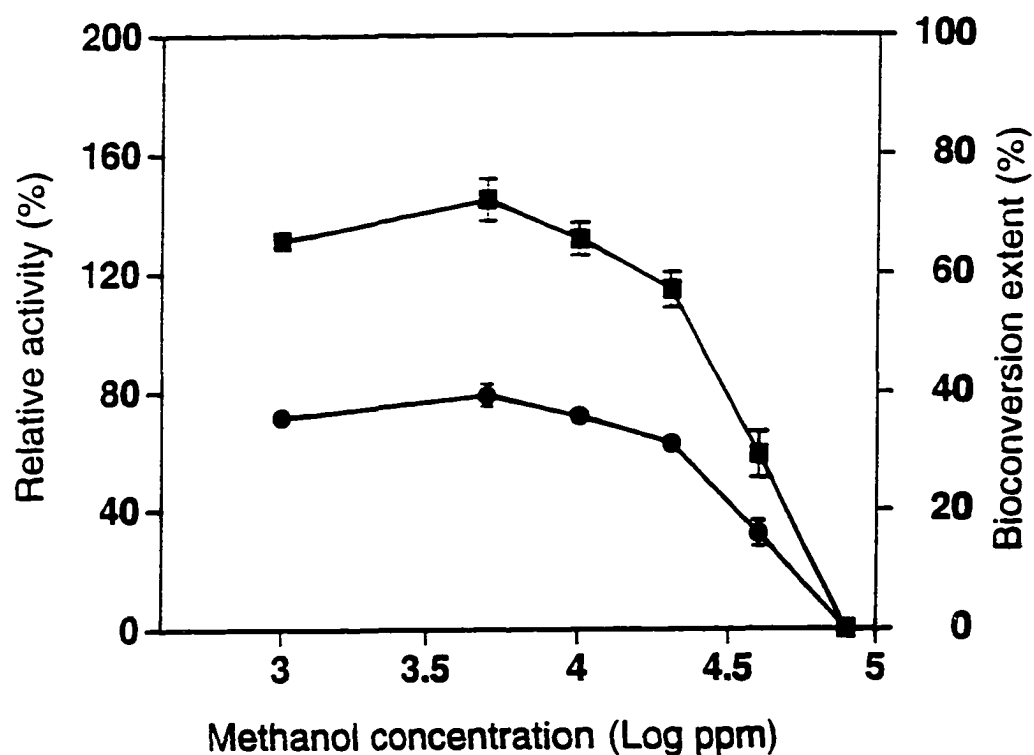


Fig. 31. Effect of methanol concentration on free cells bioconversion extent and activity relative to the control (100%) without methanol. Free cells were 12 h growing *P. digitatum* mycelia. Activity relative to control without methanol addition (close square) and bioconversion extent (closed circle) were shown. Values are an average of 3 trials. Error bars show the SD.

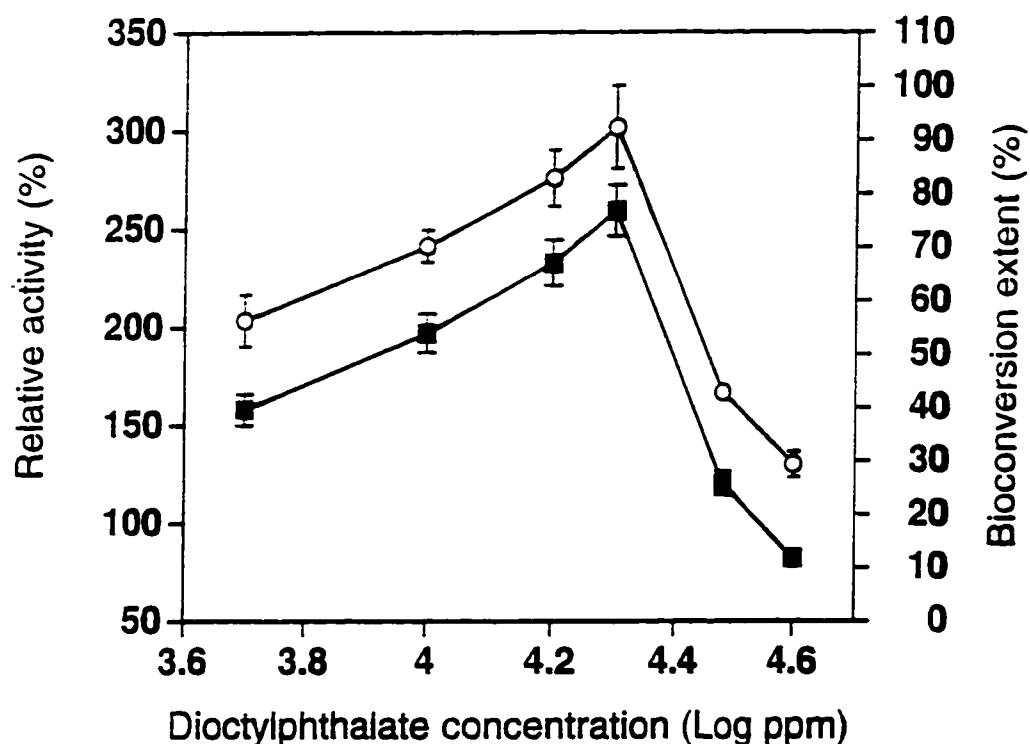


Fig. 32. Comparison of diethylphthalate concentration on free cells bioconversion extent and activity relative to the control (100%) without diethylphthalate. Diethylphthalate concentration was based on 5 ml of 12 h growing mycelia broth for each assay. Relative activity to control (closed square) and bioconversion extent (open circle) are shown. Values are an average of 3 trials. Error bars show the SD.

Surfactants (such as Tween 80, and Triton 100-X) with 0.1% increased the bioconversion yield of immobilized cells by about 2 fold. These surfactants did not affect the bioconversion when free cells were used (Table 10). Tween 80 concentrations were tested with immobilized fungal cells between 0 ppm (no Tween 80 addition as control) and 80,000 ppm (8%) (Fig. 33). Tween 80 increased bioconversion yield by immobilized cells over a range from 100 ppm to 40,000 ppm. The yield increased more than three fold when Tween 80 concentration ranged from 5,000 to 40,000 ppm. Because of potential difficulties in product recovery with high concentrations of Tween 80, 1% was chosen as the optimum concentration of Tween 80 for this bioconversion. In this case, the highest bioconversion extent was 46% when 2% of substrate concentration was used. The bioconversion activity changes with time for free and immobilized cells after 1% (v/v) Tween 80 addition were determined (Fig. 34, 35). Tween 80 showed some effect on the free cells activity during the first 24 to 36 hr. However, as time progressed, the Tween 80 showed some inhibition on the bioconversion activity (Fig 34). The effect of Tween 80 on immobilized cells was opposite (Fig 35). The discrepancy of bioconversion activities between with and without Tween 80 were highest in the first 2 days. After 2 days, the difference between the two decreased. However, the improvement of bioconversion in the presence of Tween 80 on immobilized cells was significant (Fig 35).

Primary and secondary propylene glycol increased bioconversion activity whether free or immobilized cells were used. The magnitude increase for immobilized cells ranged from 31 to 55% (Table 9). The increased yield for free cells was only 3 to 23% (Table 9).

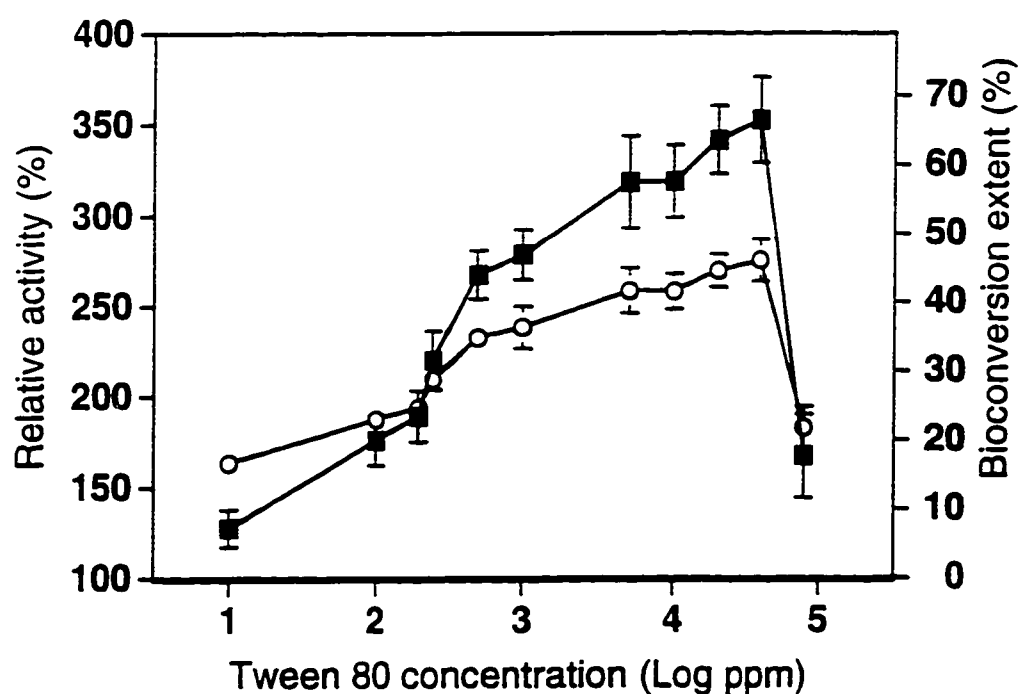


Fig. 33. Effect of Tween 80 concentration on bioconversion extent and relative activity of immobilized *P. digitatum* mycelia. Closed square is activity relative to the control (100%, no Tween 80 addition). Open circle shows bioconversion extent. Values are an average of 3 trials. Error bars show the SD.

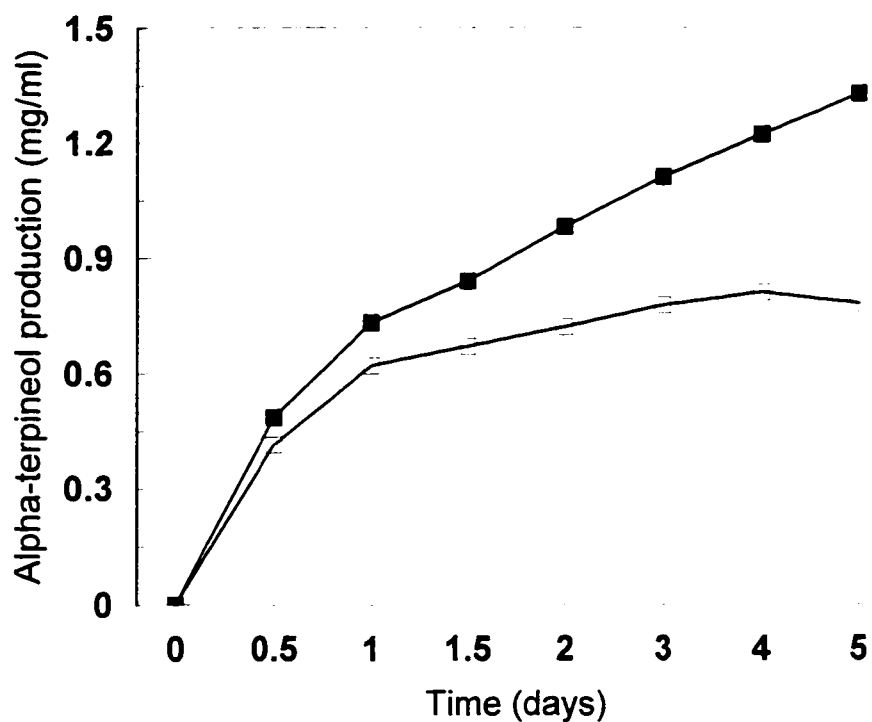


Fig. 34. Time course of α -terpineol production of free *P. digitatum* mycelia with and without Tween 80 addition. Free cells were 12 hr growing cells. Tween 80 concentration was 1% (v/v) in assay mixture. Free cells only (closed square) and with Tween 80 addition (open square) are shown.

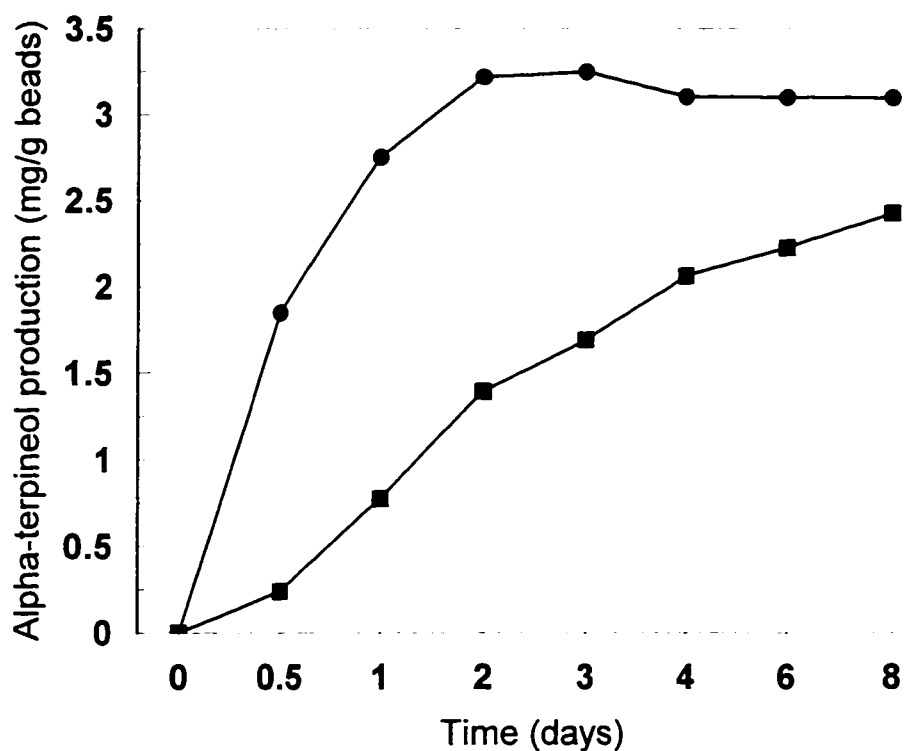


Fig. 35. Time course of α -terpineol production of immobilized *P. digitatum* mycelia with and without Tween 80 addition. Three grams of immobilized beads in 5 ml of buffer (pH 7) were used for each assay. Tween 80 concentration was 1% (v/v) based on the buffer volume. Immobilized beads with (closed circle) and without Tween 80 (closed square) are indicated.

In summary, this study clearly showed that the optima conditions for biconversion of limonene to α -terpineol with free and immobilized cells were different. For free cells, maximum conversion yields may be achieved with 1.6mg/ml D.O, 1% (v/v) limonene, pH 4.5, and the addition of 2% (v/v) of dicotylphthate reaction at 28°C for 1 day. With immobilized cells in batch form, the following conditions may be used to obtained the maximum α -terpineol yield: 1.6 mg/ml D.O, 4% of limonene, 0.05 M of pH 7 citrate-phosphate buffer solution with a 1 to 2% (v/v) of Tween 80, temperature at 28-30°C, and conversion for 3 days. Maximum yield of immobilized cells in a bioreactor with continuous feed can be obtained at 0.0144 h⁻¹ dilution rate, 0.3 SLPM of areation. and with other conditions the same as batch bioconversion.

DISCUSSION

The bioconversion of limonene to α -terpineol by *P. digitatum* was demonstrated in this study. Limonene is bioconverted to α -terpineol through a hydroxylation reaction. Biological hydroxylations by fungi are usually carried out by cytochrome P-450 dependent mono-oxygenases. It was previously assumed that this reaction occurs via epoxidation and reductive cleavage of the epoxide (Kieslich, et al., 1986). The enzyme that catalyzed the epoxidation with the bacteria *Pseudomonas oleovorans* and was suggested to be a mixed functional oxidase since both NADH and O₂ were required (May and Abbott, 1973). Cytochrome P-450 dependent mono-oxygenase enzymes were thought to be responsible for the hydroxylation of varieties of organic compounds by different fungi (Holland et al., 1982, 1988, and 1989). However, fungal hydroxylase enzymes are notoriously difficult to isolate, very few having been obtained even in crude cell-free form (Breskvar and Plevnik, 1977, Holland et al., 1988, Holland et al., 1990,). A cytochrome P-450 dependent mono-oxygenase had been successfully isolated from yeast sources. Honeck et al. (1982) purified an NADPH-cytochrome P-450 reductase from yeast *Lodderomyces elongisporus*. Hexadecane hydroxylation was catalyzed by this enzyme. The cytochrome P450 mono-oxygenase systems from *Candida maltosa* were reconstituted into *Saccharomyces cerevisiae* in vivo. The coexpressing systems showed the ability to transform lauric acid to ω -hydroxylauric acid (Zimmer et al., 1995).

There is some indirect evidence that this bioconversion was catalyzed by this cytochrome P-450 dependent mono-oxygenase. The enzyme was inhibited by

phenanthroline, an iron chelating agent (Fig. 17). Cytochrome P-450 mono-oxygenase contains flavoproteins and thus may be inhibited by this chelator. Other indirect evidence includes the inducibility of this enzyme. According to Zimmer et al. (1995), the cytochrome P-450 dependent mono-oxygenase is inducible. Inducible enzymatic activity was also reported from the liver microsomal mono-oxygenase system of animals (Orrenius and Ernster, 1974). The bioconversion activity of limonene to α -terpineol in *P. digitatum* was found only in the early log phase of the fungal growth cycle. After the mid-log phase, the bioconversion activity decreased and disappeared in late-log phase. However, the amount of bioconversion activity increased with substrate induction. This phenomenon has also been reported for other hydroxylations in several fungal systems (Schlosser and Schmauder, 1991, Holland et al., 1988).

A characteristic of the cytochrome P450 monooxygenase of yeast is that it requires low oxygen tension for production (Zimmer et al., 1995). This requirement was observed for the limonene bioconversion by *P. digitatum*. The highest bioconversion activity was obtained when *P. digitatum* was grown under low oxygen tension. For both free and immobilized cells, the highest overall activities were obtained when there was 1.6 mg/ml of oxygen in the culture. The lowest activity was found in cultures with a D.O value of 0.77 mg/ml. As might be expected, the free cell cultures were more oxygen sensitive than immobilized cells. With free cells, the activity decreased 9-fold when the oxygen tension in the culture increased from 1.6 to 3.3 mg/ml, whereas, the activity only decreased 2 fold with immobilized cells. This difference can be explained as immobilization will produce

a diffusion barrier to O₂ transfer to mycelia inside the support. It was obvious that the bioconversion required some level of O₂ and excess O₂ inhibited the bioconversion.

The pH pattern of bioconversion also indicated the nature of the enzymatic system. Cytochromes P-450 proteins are a family of isozymes capable of monooxygenase activity leading, among other reactions, to alkane hydroxylation (Ortiz de Montellano, 1986). Yang (1988) reported that different P450 isozymes from liver microsomes had different stereoselectivity on epoxide hydration of polycyclic aromatic hydrocarbons. Most microorganisms exhibit cytochrome P-450-type oxidative reactions, such as hydroxylation and N-demethylation, similar to mammalian hepatic microsomal cytochrome P-450 systems (Griffiths, et al., 1991). The progesterone, 11 α -hydroxylase system of *Rhizopus nigricans*, is made up of four proteins: cytochrome P-450, NADPH-cytochrome P-450 reductase, cytochrome b₅, and cytochrome b₅ reductase (Osborne, et al., 1990, Ghosh and Samanta, 1980, Jayanthi et al., 1982, and Madyastha et al, 1984). It is not known how many isozymes and enzyme components of cytochrome P-450 dependent mono-oxygenase there are in *P. digitatum*. The pH profile of this reaction showed four distinct peaks. This could indicate multi-protein components in the bioconversion enzyme system. Immobilization shifted the pH optimum about 0.5 pH units in the acid direction. This effect was also found when beta-glucosidase of *Aspergillus terreus* immobilized onto agar beads (Tsai, 1984). This shift may be explained by the microenvironment of the enzyme system in the cell being more basic than in the bulk solution. Besides pH shifts, this micro-environmental pH condition could

also change the activity of each protein component of the enzyme system, causing the magnitude changes observed in each peak between the free and immobilized cells.

End product inhibition of bioconversion by α -terpineol was observed. End product inhibition gave a K_{iapp} of 12.08 mM for this conversion (Fig 16). This means the velocity of reaction decreased by one half compared, to the initial velocity, when the end product accumulated to 12.08 mM in the reaction mixture. The plot also indicates that the maximum rate of this bioconversion is about 5 mg/ml (5 g/L) of α -terpineol produced per day by free cells. However, the product yields never reached this concentration. The highest product yield was about 3.2 mg/ml. There were some research reports on minimizing end product inhibition and /or toxic effects, and limiting evaporation during essential oil production by microorganisms. Several authors have employed the addition of lipophilic agents absorbing the mostly lipophilic components of the essential oils (Mattiason, 1983, Becker et al., 1984, Sprecher and Hanssen, 1985). Addition of Amberlite XAD-2 as lipophilic component adsorbed nearly 5 times as much monoterpenes that could be produced by fermentation with *Ceratocystis variispora* (Sprecher and Hanssen, 1985). Schindler (1982) produced 1.9g/L of metabolites by *C. variispora* cultivated in 20-L fermenters with gradual addition of starch by circulating the fermenter broth through an external vessel with Amberlite XAD-2. Schmauder et al. (1991) claimed that end product inhibition was not detected when they converted 13-ethyl-gon-4-en-3,17-dione (GD) to 15-OH-GD with vegetative mycelium of *Penicillium raistrickii*. Some of these approaches may be useful in this system to overcome end product inhibition.

The concentration of bioconversion product, α -terpineol, almost remained level after it reached the maximum amount. In several cases, the concentrations of α -terpineol decreased about 10 to 15% compared with the maximum amount after 8 days. This decrease was probably a result of evaporation. This result was suggested that the fungus either could not metabolize α -terpineol or metabolized it very slowly. The similar result was reported by the bioconversion of limonene with *Pseudomonas gladioli* (Cadwallader, et al., 1989).

High concentrations of organic compounds are often toxic to cells either because they are cytotoxic to cell membranes or have general anesthetic properties (Hugo, 1971, Salter and Kell, 1995). When organic substrates, such as the terpenes, are used in bioconversion systems, high substrate concentrations may be toxic to the cells themselves. Optimum substrate concentrations must be determined. The optimum substrate concentration was reported to be 0.6% (v/v) in reaction mixtures when transformation of α -pinene with six different fungi were conducted (Prema and Bhattacharyya, 1962). Their results indicated that biotransformation yields dropped sharply with further increases in substrate concentration because of toxic effects from the high substrate concentration to biocatalysts. However, bioconversion activity with growing cells in this study increased up to 1% (v/v) of substrate concentration. The bioconversion activity did not change significantly when the substrate concentration increased from 1% to 8% (v/v). Limonene toxicity to *P. digitatum* was not obvious up to an 8% substrate concentration. Substrate toxicity effect on immobilized cells was even less than on free cells since bioconversion activity with immobilized cells increased with

substrate up to 8% (v/v). Thus, it is obvious that immobilization protected cells from toxic effects of the substrate. The protective effects of immobilization can also be noted with phenanthroline inhibition. The K_{iapp} of phenanthroline to bioconversion activity with free cells was 7.4×10^{-3} mM. While the K_{iapp} for immobilized cells of phenanthroline was 2.24×10^{-1} mM. The inhibitory concentration of phenanthroline to the bioconversion of limonene with free cells was 30-fold less than that of immobilized cells.

Immobilization increased temperature stability. The temperature optimum was 28-32°C for free cells and 28-36°C for immobilized cells. Above 36°C for free cells, the enzyme inactivated; immobilized cells withstand this temperature for at least 1 day. Similar results have been reported on temperature stabilities of other free and immobilized enzymes (Gaikwad and Deshpande, 1992).

Immobilization of *P. digitatum* changed its affinity for the substrate. The V_{max} was not affected by immobilization but the K_m (app.) of immobilized cells, increased 40%. The K_m (app) increase was probably caused by a decrease in the availability of substrate to the enzyme system. Bioconversion extent was also lowered for immobilized cells. A K_m (app) of α -terpineol dehydratase (α -TD), an enzyme isolated from *P. gladioli* which has been used for conversion of limonene to α -terpineol, was determined as 2.18 ± 0.19 mM (Cadwallader, et al., 1992). This is quite different from the K_{mapp} of this fungal enzyme system. Obviously, this fungal enzyme system is a different system from the α -TD isolated from the bacterium. This conclusion has been previously assumed by Braddock and Cadwallader (1995).

The bioconversion conducted by this system showed strict stereoselectivity and stereospecificity. The enzyme system in *P. digitatum* only converted (4R)-(+)-limonene to (4R)-(+)- α -terpineol. (4S)-(-)-limonene was not be converted. This is an advantage for the production of pure (4R)-(+)- α -terpineol. Similar stereochemical properties of this fungus were reported by Stumpf et al. (1982) when they used it for the bioconversion of racemic forms of limonene.

The substrate used in this research is a lipophilic compound with a low solubility in an aqueous media. The following strategies were applied to increase the reaction rates: (1). addition of water-organic co-solvent systems (methanol, dimethyl-sulfoxide, propylene glycol, glycerol, and several esters); (2) addition of non-ionic surfactants (Tween 80 and Triton 100-X); and (3) the use of organic solvent systems. Methanol at 0.5% (v/v) was found to improve the yields of this bioconversion with free cells. At this concentration, the α -terpineol yield increased about 50%. When the methanol concentration was greater than 2.5% (v/v), the yield rapidly decreased. This critical point effect exists for many organic co-solvents (Salter and Kell, 1995). Methanol, as a water-miscible organic solvent, has been used in steroid bioconversions (Ohlson, et al., 1980, Fukui and Tanaka, 1984). In general, all of the water-organic co-solvents tested in this experiment produced some positive effect. The most significant yield improvement was from the two water-organic co-solvents esters dioctylphthalate and ethyl decanoate. They increased product yield by 2.7 and 2.2 fold, respectively. Lilly et al. (1987) also indicated that these two solvents and dodecane (all of them Log P > 4) improved the rates of Δ^1 -dehydrogenation of hydrocortisone by *Arthrobacter simplex*. The optimum concentration

of dioctyl phthalate was determined to be about 2% (v/v) for this bioconversion, whereas Lilly et al. (1987) used equal amounts of organic and aqueous phases. In their biotransformation, the conversion rates were tested within 2 hrs of reaction. Bioconversion of limonene by *P. digitatum* takes more than 12 hrs. Exposure time is an important factor influencing the cytotoxicity of organic solvents (Salter and Kell, 1995).

The non-ionic surfactants like Tween 80 and Triton X-100, significantly improved the bioconversion yields of immobilized *P. digitatum* mycelia, but had no effect on free cells. The support Ca-alginate is hydrophilic and the substrate is lipophilic. It may have been difficult for immobilized cells to come in contact the substrate. Studies have been performed on the comparison of bioconversion activity using hydrophilic (H-gel) and lipophilic (l-gel) gels to immobilize microorganisms (Omata et al., 1979, Yamane et al., 1979, and Fukui et al., 1980). In transformations of highly hydrophobic substrates, as steroids, terpenoids, and various organic compounds with immobilized microbial cells or enzymes, and the use of the highly lipophilic character of gel-entrapped biocatalysts are more effective reaction systems. The solubility of the substrates as well as the products is significantly enhanced and the hydrophobicity of the gels makes their diffusion through gel matrices much easier. In this study, the addition of non-ionic surfactants was tested. After addition of non-ionic surfactants, the solubility of substrates increased and enhanced the chances of contact with the cells were also increased. A 1% concentration of Tween 80 with immobilized mycelium is recommended for this bioconversion even though the highest activity was obtained from a 4% Tween 80 concentration, because of product recovery and downstream process consideration. There were no significant

differences in product yields between 1 to 4% (v/v) of Tween 80. Activity decreased when the Tween 80 concentrations were more than 4%. This may not be inhibition, but rather a loss in product recovery at this high concentration. Tween 80 had no effect on bioconversion with free cells. A possible explanation for this is the nature of the lipophilic walls of vegetative mycelia. Even without addition of the detergent, the surrounding mycelia may already be saturated with the substrate. A non-ionic surfactant, Praxo W-100, was reported to enhance the activity of the hexadecane hydroxylation, which was catalyzed by the purified NADPH-cytochrome P-450 reductase from the yeast *Lodderomyces elongisporus* (Honech et al., 1982). Tween 80 was used as a surfactant to accelerate cholesterol degradation rates with *Mycobacterium* strain DP (Smith et al., 1993).

Organic solvent systems were less effective than water-miscible co-solvent systems or non-ionic surfactants. Isooctane has been used to enhance the microbial conversion of β -ionone with *Aspergillus niger* (Sode et al., 1989). This organic solvent showed a negative effect on limonene bioconversion by *P. digitatum*. Methanol is toxic to β -ionone conversion, but enhanced limonene bioconversion. The effects of hexane on both conversions were similar. Some authors have suggested that the Log P value may help to choose organic solvent for two-phase systems (Lanne et al., 1987 a, b, Osborne et al., 1992). Solvents with a log P value above 4 generally are very hydrophobic and show no toxic effects on biocatalysts (Lanne et al. 1987, and Osborne et al., 1990, Sonsbeek, et al., 1993). However, this rule seems not to completely hold true for the solvents used in this bioconversion. The log P values of both isooctane and tetradecane are above 4, but

the relative activities were 55% and 34%, respectively compared with the no solvent addition control (Table 8). Although the log P of both dioctyl phthalate and ethyl decanoate are above 4, and they enhanced this bioconversion. Halling (1994) pointed out that when to use or not use log P is dependent on mechanisms used for the applications. Log P value is an appropriate parameter to measure the tendency of solvents or other organic molecules to partition between phases of differing polarity. However, if other mechanisms involve the direct effects of the bulk organic phase, or the solvation of other species within in it, then, log P of the solvent is not an appropriate parameter.

Using immobilized cells in repeated batches, was tested for the reusability of the immobilized cells. It was found that the beads resuability was not as good as reported for the bioconversion of 15 α -hydroxylation of 13-ethyl-gon-4-ene-3,17-dione conducted by Schmauder et al. (1991). The average productivity of limonene bioconversion in the second run was about one third of that of the first run. The third run had only 6% of productivity of the first run. The beads were regenerated after the third use and the productivity of the fourth and fifth runs increased but the productivity of those beads only reached a level achieved in the second run. In contrast, the bioconversion of 13-ethyl-gon-4-en-3,17-dione (GD) to 15-OH-GD with immobilized *P. raistrickii* on calcium alginate beads kept the relative activity at 100% even after 10 runs with beads regenerated every several run (Schlosser and Schmauder, 1991). The reusability of immobilized beads may be related to the characteristics of the bioconversion as well as to the properties of physical strength of the beads. The resuablity of beads has been tested by Vidyarthi and Nagar (1994) for use of bioconversion of progesterone with *Rhizopus nigricans*. Their

bioconversion productivity dropped about 60% to 30% after the 4th run for three different supporting materials (agar gel, polyacrylamide and chitosan). Similar results showed up in this study, as they found that the relative percent conversion decreased as the number of the usage increased.

As indicated previously, the enzyme involved in this bioconversion was inhibited by a high oxygen levels. Aeration not only influenced the productivity but also the half-life of the immobilized beads. The highest productivity at two flow rates (0.0072 and 0.0144 h⁻¹) was obtained from 0.3 SLPM aeration. The half-life of the immobilized cells for both cases was about 6 to 7 days. In all runs with continuous feed, the longest half-life was found with 0.2 SLPM aeration and 0.0072 h⁻¹ dilution rate. The half-life was estimated to be about 20 days. There seems to be a negative relationship between the aeration and half-life of the immobilized cells. This may be related to the inhibition of this bioconversion at higher oxygen tension. The average of daily relative productivity compared with the control was calculated. It was concluded that 0.3 SLPM was the best aeration rate for the highest productivity. A flow rate of 0.0144 h⁻¹ was better than a flow rate 0.0072 h⁻¹ at this aeration rate.

Current commercial production of α -terpineol is through the hydration of pinene or turpentine oil with aqueous mineral acid. According to the recent Chemical Marketing Reporter, the price of α -terpineol is \$2.17 per pound. The limonene price in current market is \$1.30 per pound. Since the α -terpineol produced through this process is an optically pure and can be labelled as a natural flavor compound instead of artificial, it is estimated that the price of (4R)-(+)- α -terpineol produced by this process would be around

\$7-10 per pound. In this study, the α -terpineol yield was about 3.2 mg/ml (3.3 g/L) with growing cells. This yield is the highest α -terpineol yield by microbial biotransformations that has been reported to date. The maximum α -terpineol concentration obtained from bioconversion of limonene by *Pseudomonas gladioli* was 702 ppm (Cadwallader et al., 1989). α -Terpineol yield from *Cladosporium species* bioconversion of limonene was 1 g/L (Kraidman et al., 1969). However, the minimal medium cost for production of one pound of α -terpineol through this bioconversion is about \$33.40, which is higher than the amount selling price of the product. Therefore, this process in general is not profitable under current yield levels and the cost of the chemicals. α -Terpineol produced through this bioconversion was optical pure (4R)-(+)- α -terpineol. There is no pure (4R)-(+)- α -terpineol available in the market presently. Thus, under special conditions, this process may be applicable. It was estimated that industrial production of a single flavor substrate by microorganisms only became economical if the compound had a market value of \$200/kg (Welsh et al., 1989). This constraint has delayed applied research in this area. Therefore, the microbial transformation of terpenoid compounds is only of potential interest for practical application in the flavor and fragrance industry under current conditions.

SUMMARY

- *P. digitatum* stereospecifically transferred (R)-(+)-limonene to (4R)-(+)- α -terpineol.
This bioconversion was only found in early-log phase of fungal growth cycle.
- Oxygen was found to be critical. Too little or oxygen affected the activity.
- Temperature optimum of free and immobilized cells was 28°C.
- pH optimum of free and immobilized cells was pH 4.5 and pH 7.0 respectively.
- Bioconversion with free cell took less than that of immobilized cells.
- Maximum of α -terpineol form this bioconversion could be around 5 mg/ml by using free cells.
- Water-miscible organic co-solvents (dioctylphthalate) improved the yield of this bioconversion with free cells.
- Non-Ionic surfactants (Tween 80, Triton 100-X) improved the bioconversion yield with immobilized cells.
- Immobilization of the *P. digitatum* into calcium alginate beads for continuous operation is possible. The half-life of these beads in the bioreactor ranged from 7 to 20 days dependent on the rate of aeration.
- The activity of immobilized beads was not affected for at least 14 days when they are stored at 4°C.
- The enzyme catalyzed this bioconversion was believed to be an inducible cytochrome P-450 dependent monooxygenase.
- Application of this process to industrial production may not be possible in the current market.

REFERENCES

- Abraham, W.R., Stumpf, B., Kieslich, K., Reif, S., and Hoffmann, H.R., 1986. Biotransformation of tetramethyl-limonene. *Appl. Microbiol. Biotechnol.* 24:31-34.
- Abraham, W.R. 1994. Phylogenetic influences in microbial hydroxylation of terpenoids. *World Journal of Microbiology & Biotechnology*, 10: 88-92.
- Abraham, W.R., Ernst, L., Stumpf, B., and Arfmann, H.A. 1989. Microbial hydroxylations of bicyclic and tricyclic sesquiterpenes. *J. Ess. Oil Res.*, 1:19-27.
- Ahmad S., and Johri, N., 1992. Immobilization of *Rhodococcus equi* DSM 89-133 onto porous celite beads for cholesterol side-chain cleavage. *Appl Microbiol Biotechnol*, 37:468-469.
- Allegrone, G., Barbeni, M., Cardillo, R., Fuganti, C., Grasselli, P., Pisciotta, A. 1991. On the enantiomeric composition of biogenerated oct-1-en-3(R)-ol. *Biotechnology Letters*, 13 (10): 711-714.
- Andersson, E., and Hahn-Hagerdal, B. 1990. Bioconversions in aqueous two-phase systems. *Enzyme Microb. Technol.*, 12: 242-254.
- Anon, 1990. Chemical marketing reporter, Van, H; Deyrup, C.A.; and McCoy, M. (Eds), Schnell Publishing Co., Ltd. New York, 238 (12): 38, 40, 44.
- Arctander, S., 1969. Perfume and flavor chemicals, Chapter two, Montclair, NJ.
- Asakawa, Y., Takahashi, H., Toyota, M., and Noma, Y. 1991. Biotransformation of monoterpenoids, (-)-and (+)-menthols, terpinolene and carvotanacetone by *Aspergillus species*. *Phytochemistry*, 30 (12): 3981-3987.
- Azerad, R., 1993. Microbiological hydroxylations: myths and realities. *Chimia*, 47: 93-96.
- Bailey, M.J., Paoutanen, K. 1989. Production of xylanolytic enzymes by strains of *Aspergillus*. *Appl Microbiol Biotechnol.*, 30: 5-10.
- Bally, N.R., Bhattacharyya, P.K, and Rangachari, P.N. 1968. Microbiological transformations of terpenes: Part XIV- Purification & properties of perillyl alcohol dehydrogenase. *Indian J. Biochem.*, 5: 1-6.

- Bauer, K. and Garbe, D., 1985. Common fragrance and flavor materials, preparation, properties and uses, VCH Verlagsgesellschaft mbH, D-6940 Weinheim (Federal Republic of Germany).
- Becker, H., Reichling, J., Bisson, W., Herold, S. 1984. Two phase culture - a new method to yield lipophilic secondary products from plant suspension cultures. Third European Congress on Biotechnology. 1: 209-213.
- Bhattacharyya, P.K. and Ganapathy, K. 1965. Microbiological transformations of terpenes: Part VI. Studies on the mechanism of some fungal hydroxylation reactions with the aid of model systems. Indian J. Biochem., 2:137-145
- Bhattacharyya, P.K., Prema, B.R., Kulkarni, B.D. and Pradhan, S.K. 1960. Microbiological transformation of terpenes: hydroxylation of α -pinene. Nature 187: 689-690.
- Boeren, S., Laane, C., and Hilhorst, R. 1987. Viability and activity of *Flavobacterium dehydrogenans* in organic solvent/culture two-liquid-phase-systems. In: Biocatalysis in Organic Media. Lanne, C., Tramper, J., and Lilly, M.D., Eds., Elsevier, Amsterdam, pp 303-310.
- Bon, E., Webb, C. 1989. Passive immobilization of *Aspergillus awamori* spores for subsequent glucoamylase production. Enzyme Microb Technol., 11: 495-499.
- Braddock, R. J., and Cadwallader, K.R., 1995. Bioconversion of citrus d-limonene, Fruit flavors, Rouseff, R. L. and Leahy, M.M. (Eds.), ACS Symposium Series 596. pp 142-148.
- Brand, J.M., Brache, J.W., Markobetz, A.J., Wood, D.L. and Browne, L.E. 1975. Production of vebenol pheromone by a bacterium isolated from bark beetles. Nature 245: 136.
- Breskvar, K. and Plevnik, T. P., 1977. A possible role of cytochrome P-450 in hydroxylation of progesterone by *Rhizopus nigricans*, Biochem. Biophys. Res. Commun: 74: 1192-1198.
- Brink, L.E.S., and Tramper, J. 1985. Optimization of organic solvent in multiphase biocatalysis, Biotechnol. Bioeng., 27: 1258-1269.
- Cadwallader, K.R., Braddock, R.J. 1992. Enzymatic hydration of (4R)-(+)-limonene to (4R)-(+)- α -terpineol. In Development in Food Science, Vol. 129: Food Science and Human Nutrition. G. Charambous (Ed.) Elsevier Science Publishers B.V., New York, pp. 571-584.

- Cadwallader, K.R., Braddock, R.J., and Parish, M.E. 1992. Isolation of α -terpineol dehydratase from *Pseudomonas gladioli*. J. Food Science, 57 (1): 241-248.
- Cadwallader, K.R., Braddock, R.J., Parish, M.E., and Higgins, D.P. 1989. Bioconversion of (+)-limonene by *Pseudomonas gladioli*. J. Food Sci. 54:1241-1245.
- Cardini, G., and Jurthuk, P., 1970. The enzymatic hydroxylation of n-octane by *Corynebacterium* sp. Strain 7E1C, The Journal of Biological Chemistry, 245 (11): 2789-2796.
- Ceen, E.G., Herrmann, J.P.R., and Dunnill, P. 1987. Solvent damage during immobilized cell catalysis and its avoidance: studies of 11 α -hydroxylation of progesterone by *Aspergillus ochraceus*. Appl Microbiol Biotechnol. 25: 491-494.
- Chibata, I. 1978. Immobilized enzymes. John Wiley & Sons, Inc. New York, pp 73-79.
- Colby, J. and Dalton, H. 1976. Some properties of a soluble methane mono-oxygenase from *Methylococcus capsulatus* Strain Bath. Biochem. J., 157: 495-497.
- Colby, J., Stirling, D. E., and Dalton, H., 1977. The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath) its ability to oxygenate n-alkanes, n-alkenes, ethers, and alicyclic, aromatic and heterocyclic compounds, Biochem. J. 165:395-402.
- Croteau, R., 1980. The biosynthesis of terpene compounds. In: Fragrance and Flavor Substances. R. Croteau (Ed.), D & PS. Verlag, 3017 Pattensen 1, W. -Germany, pp 13-36.
- Devi, J.R. and Bhattacharyya, P.K. 1977. Microbiological transformations of terpenes: Part XXIII. Fermentation of geraniol, nerol and limonene by soil pseudomonad, *Pseudomonas incognita* (Linalool strain). Indian J. Biochem. Biophys. 14: 288-291.
- Devi, J.R. and Bhattacharyya, P.K. 1977. Microbiological transformations of terpenes: Part XXIV: Pathway of degradation of linalool, geranoil, nerol & limonene by *Pseudomonas incognita* (linalool strain), Indian J. Biochem. Biophys. 14:359-372.
- Dhavalikar, R.S.; and Bhattacharyya, P.K. 1966a, Microbiological transformation of terpenes: Part VIII Fermentation of limonene by a soil *Pseudomonas*. Indian J. Biochem. 3: 144-157.
- Dhavalikar, R.S. Rangachari, P.N., and Bhattacharyya, P.K. 1966b. Microbiological transformation of terpenes: Part IX. Pathways of degradation of limonene in a soil *Pseudomonad*. Indian J. Biochem. 3: 158-164.

- Dhavlviakar, R.S., Ehbreecht, A. and Albrioscheit, G. 1974. Microbial transformations of terpenoids: β -pinene. Dragoco Report 3: 47-49.
- Draczynska, B., Cagara, C., Siewinski, A., Rymkiewicz, A., Zagza, A., and Leufven, A., 1985. Biotransformation of pinene XVII. Transformation of α - and β -pinenes by means of *Armillariella mellea* a parasite of woodlands. J. Basic Microbiol. 25: 487-492.
- Draczynska-Lusiak, B. and Shewinski, A. 1989. Enantioselectivity of the metabolism of some monoterpenic components of coniferous tree resin by *Armillariella mella*. J. Basic Microbiol. 29: 269-275.
- Drawert F., 1988, Bioflavor-What does it mean? In Bioflavour'87, Peter Schreier (Ed.), Walter de Gruyter, Berlin and New York, pp 3-32
- Eikmeier, H., Rehm, H.J. 1984. Production of citric acid with immobilized *Aspergillus niger*. Appl Microbiol Biotechnol, 20: 365-370.
- Eikmeier, H., Rehm, H.J. 1987. Stability of calcium-alginate during citric acid production of immobilized *Aspergillus niger*. Appl Microbiol Biotechnol, 26: 105-111.
- Eramn, W.F., 1985, Chemistry of the monoterpenes: an encyclopedic handbook, part A, Marcel Dekker, Inc. pp 1-31.
- Erickson, R. E., 1976. The industrial importance of monoterpenes and essential oils, Lloydia, 39: 8-19.
- Faber, K., 1992. Biotransformations in Organic Chemistry, Springer-Verlag, Berlin Heidelberg.
- Fernandes, P., Cabral, J.M.S., and Pinheiro, H.M. 1995. Bioconversion of a hydrocortisone derivative in an organic-aqueous two-liquid-phase system. Enzyme Microb. Technol., 17: 163-167.
- Fordham, W. D., 1968. Reports on the progress of Applied Chemistry, pp. 264-273.
- Fujii, N., Yasuda, K., and Sakakibara, M. 1994. Effect of volume ratio of cellulose carriers and time interval of repeated batch culture on citric acid productivity by immobilized *Aspergillus niger*. J. Ferment. Bioeng., 78 (5): 389-393.
- Fukui, S., Ahmed, S. A., Omata, T., and Tanaka, A., 1980. Bioconversion of lipophilic compounds in non-aqueous solvent. Effect of gel hydrophobicity on diverse conversions of testosterone by gel-entrapped *Nocardia rhodocrous* cells, European J. Appl. Microbiol. Biotechnol. 10: 289-301.

- Fukui, S. and Tanaka, A., 1984. Application of biocatalysts by prepolymer methods. In: Adv. Biochem. Eng. Biotechnol. 29, (Fiechter, A., Ed), Akademie-Verlag Berlin, pp. 1-33.
- Fukui, S. and Tanaka, L.W. 1982. Immobilized cells. Ann. Rev. Microbiol., 36:145-172.
- Fukui, S., and Tanaka, A. 1984. Applications of biocatalysts immobilized by pre-polymer methods. Adv. Biochem. Eng. Biotechnol., 29:1-33.
- Fukui, S., Tanaka, A., and Iida, T. 1987. Immobilization of biocatalysts for bioprocesses in organic solvent media, In: Biocatalysis in Organic Media. Lanne, C., Tramper, J., and Lilly, M.D., Eds., Elsevier, Amsterdam, pp 21-41.
- Gaikwad, S.M. and Deshpande, V.V. 1992. Immobilization of glucose isomerase on Indion 48-R. Enzyme Microb. Technol., 14: 855-858.
- Gatfield, I. L., 1988, Production of flavor and aroma compounds by biotechnology, Food Technology, 42 (10): 110-122.
- Ghosh, D. and Samanta, T. B. 1980. J. Steroid Biochem. 14: 1063-1067.
- Gibbon, G.H., Millis, N.F. and Pirt, S.J. 1972. The degradation of α -pinene by bacteria. Proc. Int. Ferment. Symp., 4th. pp. 609-612.
- Ginbbon, G.H. and Pirt, S.J. 1971. The degradation of α -pinene by *Pseudomonas* PX I. FEBS Lett. 18: 103-105.
- Griffiths, D., Best, D.J., and Jezequel, S.G. 1991. The screening of selected microorganisms for use as models of mammalian drug metabolism. Appl Microbiol Biotechnol, 35: 373-381.
- Griffiths, E.T., Bociek, S.M., Harries, P.C., Jeffcoat, R., Sissons, D.J., and Trudgill, P.W. 1987a. Bacterial metabolism of α -pinene: Pathway from α -pinene oxide to acyclic metabolites in *Nocardia* sp. strain p18.3. J. Bacteriol. 169: 4972-4979.
- Griffiths, E.T., Harries, P.C., Jeffcoat, R., and Trudgill, P.W. 1987b. Purification and properties of α -pinene oxide lyase form *Nocardia* sp. strain P18.3. J. Bacteriol. 169: 4980-4983.
- Groboillot, A, Boadi, D.K., Poncelet, D., and Neufeld, R.J., 1994. Immobilization of cells for application in the food industry, Critical Reviews in Biotechnology, 14 (2):75-107.

- Halling, P.J. 1994. Thermodynamic predictions for biocatalysis in nonconventional media: Theory, tests, and recommendations for experimental design and analysis. *Enzyme Microb. Technol.*, 16: 178-206.
- Hanson, J.R. 1972. Introduction and nomenclature, *Chemistry of Terpenes and Terpenoids*, Newman, A.A (Ed.), Academic Press, London and New York, pp 1-10.
- Hocknull, M.D., and Lilly, M.D. 1987. The Δ^1 -dehydrogenation of hydrocortisone by *Arthrobacter simplex* in organic-aqueous two-liquid phase environments, In: *Biocatalysis in Organic Media*. Lanne, C., Tramper, J., and Lilly, M.D., Eds., Elsevier, Amsterdam, pp 393-398.
- Hofmann-La Roche, 1978. Belg. Pat. 858326. Microbiological hydroxylation of patchouli alcohol - to give intermediates for nor-patchouli alcohol synthesis.
- Holand, H.L., Chernishenko, M.J., Conn, M., Munoz, A., Manoharan, T.S., and Zawadski, M.A. 1990. Enzymic hydroxylation and sulfoxidation of cyclopropyl compounds by fungal biotransformation. *Can. J. Chem.* 68: 696-670.
- Holland, H.L., and Riemland, E. 1985. Microbial hydroxylation of steroids. 10. Rearrangement during epoxidation and hydroxylation, and the stepwise nature of these enzymic reactions. *Can. J. Chem.* 63: 1121-1126.
- Holland, H. L., and Auet, B.J. 1975. The mechanism of the microbial hydroxylation of steroids. Part 2. Hydroxylation of a Δ^4 -3-ketosteroid analog by *Rhizopus arrhizus* ATCC 11145. *Can. J. Chem.* 53: 2041-2044.
- Holland, H. L., Brown, F.M., Munoz, B., and Ninniss, R.W. 1988. Side chain hydroxylation of aromatic hydrocarbons by fungi. Part 2. Isotope effects and mechanism. *J. Chem. Soc. Perkin Trans. II*, pp 1557-1563.
- Holland, H.L., Munoz, B., and Jones, T.B. 1988. Side chain hydroxylation of aromatic compounds by fungi. 3. Direct observation of fungal biotransformation by NMR. *Bioorganic Chemistry* 16: 388-393.
- Holland, H.L., Bergen, E.J., Chenchiah, P.C., Khan, S.H., Munoz, B., Ninniss, R.W., and Richards, D. 1987. Side chain hydroxylation of aromatic compounds by fungi. 1. Products and Stereochemistry. *Can. J. Chem.* 65: 502-507.
- Holland, H.L., and Auet, B.J. 1975. The mechanism of the microbial hydroxylation of steroids. Part 2. Hydroxylation of a Δ^4 -3-ketosteroid analog by *Rhizopus arrhizus* ATCC 11145. *Can. J. Chem.* 53: 2041-2044.

- Holland, H.L., and Riemland, E. 1985. Microbial hydroxylation of steroids. 10. Rearrangement during epoxidation and hydroxylation, and the stepwise nature of these enzymic reactions. *Can. J. Chem.* 63: 1121-1126.
- Holland, H.L., Brown, F.M., and Conn, M. 1990. Side chain hydroxylation of aromatic compounds by fungi. Part 4. Influence of the para substituent on kinetic isotope effects during benzylic hydroxylation by *Mortierella isabellina*. *J. Chem. Soc. Perkin Trans. 2*: 1651-1655.
- Holland, H.L., Carter, I.M., Chenchiah, P.C., Khan, S.H., Munoz, B., Ninniss, R.W., and Richards, D. 1985. Fungal hydroxylation of ethyl benzene and derivatives. *Tetrahedron Letters*, 26 (52): 6409-6412.
- Holland, H.L., Kindermann, M., Kumaresan, S., and Stefanac, T. 1993. Side chain hydroxylation of aromatic hydrocarbons by fungi. Part 5. Exploring the benzylic hydroxylase of *Mortierella isabellina*. *Tetrahedron Asymmetry* 4 (6): 1353-1364.
- Holland, H.L., Kindermann, M., Kumaresan, S., and Stefannac, T. 1993. . Side chain hydroxylation of aromatic hydrocarbons by fungi. Part 5. Exploring the benzylic hydroxylase of *Mortierella isabellina*. *Tetrahedron: Asymmetry*, 4 (6): 1353-1364.
- Honeck, H., Schunck, W.-H., Riege, P., and Muller, H.-G. 1982. The cytochrome P-450 alkane monooxygenase system of the yeast *Lodderomyces elongisporus*: purification and some properties of the NADPH-cytochrome P-450 reductase. *Biochem. Biophys. Res. Commun.* 106: 1318-1324.
- Hugo, W.B. 1971. *Inhibition and Destruction of the Microbial Cell*. Academic Press, London.
- Inoue, A., and Horikoshi, K. 1991. Estimation of solvent-tolerance of bacteria by the solvent parameter Log P. *J. Ferm. Bioeng.*, 71: 194-196.
- Janssens, L., De Pooter, H.L., Schamp, N.M., and Vandamme, E.J., 1992, Production of flavors by microorganisms, *Process Biochemistry* 27 (4):195-215.
- Jayanthi, C. R., Madyastha, P. and Madyastha, K. M. 1982. *Biochem. Biophys. Res. Commun.* 106: 1262-1268.
- Jogelkar , S.S. Vora, M.A., Dhere, S.G., and Dhavlikar, R.S. 1968. Microbial transformations of terpenoids: Citronellal, citral, eugenol, Δ^3 -carene, α -pinene and longifolene. *I.O.& S.J.*, October, pp. 85-87.

- Jogelkar, S.S. and Dhavlikar, R.S. 1969. Microbial transformation of terpenoids. I. Identification of metabolites produced by a pseudomonad from citronellal and citral. *Appl. Microbiol.* 18: 1084-1087.
- Jones, J.B. Sih, C.J., and Perlman, D., 1976, Procedures useful in studying microbial transformations of organic compounds. *Applications of Biochemical Systems in Organic Chemistry*, Weissberger, A. (Ed.), John Wiley & Sons, New York. pp 47-68
- Kempler, G.M., 1983, Production of flavor compounds by microorganisms, *Advances in Applied Microbiology*, 29: 29-51.
- Kieslich, K., Abraham, W.R., Stampt, B., and Washausem, P., 1985. Microbial transformation of terpenoids, *Topics in Flavor Research*, H. Eichhorn. pp 406-425.
- Kieslich, K., Abraham, W.R., Stumpf, B., Thede, B., and Washausen, P. 1986. Transformations of terpenoids. In *Progress in Essential Oil Research*, Brunke, E.J. (Ed.), Walter de Gruyter & Co. Berlin, New York, pp 367-394.
- Koshcheyenko, K.A., Turkina, M.V., Skryabin, G. K. 1983. Immobilization of living microbial cells and their application for steroid transformations. *Enzyme Microb. Technol.* 5: 14-28.
- Krabnobajew, V., Helmlinger, D. 1982. Fermentation of fragrances: biotransformation of β -ionone by *Lasiodiplodia theobromae*. *Helv. Chim. Acta* 65: 1590-1601.
- Kraidman, G., Mukherjee, B.B., Hill, I.D., 1969. Conversion of D-limonene into an optically active isomer of α -terpineol by a *Cladosporium species*. *Bacteriological Proceedings*, pp. 63.
- Krasnobajew, V., (Givaudan), US Pat. 4311860 Prior. Date Nov. 24, 19878.
- Krasnobajew, V., and Helmlinger, D. 1982. Fermentation of fragrances: biotransformation of β -ionone by *Lasiodiplodia theobromae*, *Helvetica Chimica, Acta.* 65:1590-1601.
- Kumar, P.K.R., and Lonsane, B.K. 1988. Immobilized growing cells of *Gibberella fujikuroi* P-3 for production of gibberellic acid and pigment in batch and semi-continuous cultures. *Appl Microbiol Biotechnol.* 28: 537-542.
- Kutney, J.P., Berset, J-D., Hewitt, G.M., and Singh, M. 1988. Biotransformation of dehydroabietic, abietic, and isopimaric acids by *Mortierella isabellina* immobilized in polyurethane foam. *Appl. Environ. Microbiol.* 54: 1015-1022.

- Laane, C., Boeren, S., Hilhorst, R., and Veeger, C. 1987b. Optimization of biocatalysis in organic media, In: *Biocatalysis in Organic Media*. Lanne, C., Tramper, J., and Lilly, M.D., Eds., Elsevier, Amsterdam, pp. 65-84.
- Laane, C., Boeren, S., Vos, K., and Veeger, C. 1987a. Rules for optimization of biocatalysis in organic-solvents. *Biotechnol. Bioeng.*, 30: 81-87.
- Lanza E. and Palmer, J.K. 1977. Biosynthesis of monoterpenes by *Ceratocystis moniliformis*. *Phytochemistry*, 16: 1555-1560.
- Larroche, C., Creuly, C., Gros, J. -B. 1995. Fed-batch biotransformation of β -ionone by *Aspergillus niger*. *Appl. Microbiol Biotechnol* 43: 222-227.
- Layman, P.L. 1984. Flavors and fragrances industry faces season of consolidation. *Chem. and Eng.*, 30: 7-13.
- Leufven, A., Bergstorm, G. and Falsen, E. 1988, Oxygenated monoterpenes produced by yeasts, isolated from *Ips ypographus* and grown in phloem medium. *J. Chem Ecol.* 14: 353-359.
- Lilly, M. D., Brazier, A. J., Hocknull, M. D, Williams, A.C, and Woodley, J. M. 1987. Biological conversions involving water-insoluble organic compounds. In *Biocatalysis in Organic Media*. Lanne, C., Tramper, J., and Lilly, M.D., Eds., Elsevier, Amsterdam, pp 3-17.
- Lineweaver, H. and Burk, D. 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* 56: 658-666.
- Loomis, W.D. 1967. Biosynthesis and metabolism of monoterpenes. In "Terpenoids in Plants". J.B. Pridham (Ed.). Academic Press Inc., New York. pp. 59-82.
- Lusiak, B.D., and Siewinski, A. 1989. Enantioselectivity of the metabolism of some monoterpenic components of coniferous tree resin by *Armillariella mellea* (honey fungus). *J. Basic Microbiol.* 29 (5): 269-275.
- Madyastha, K.M., Jayanthi, C.R., Madyastha, P., and Sunathi, D. 1984. *Can. J. Biochem. Cell. Biol.* 62: 100-107.
- Mahato, S. and Majumdar, I., 1993. Current trends microbial steroid biotransformation. *Phytochemistry*, 34 (4): 883-898.
- Mahato, S.B., and Majumdar, I. 1993. Current trends in microbial steroid biotransformation. *Phytochemistry*, 34 (4): 883-898.

- Manley, C.H., 1987, Processing and biotechnology as source of flavors, Perfume and Flavorist, Vol 12, August/September: 11-22.
- Manolov, R. J. 1992. Batch and continuous ribonuclease production by immobilized *Aspergillus clavatus* cells in a bubble-column bioreactor. Appl Microbiol Biotechnol, 37: 32-36.
- Manson, H.S., 1957. Mechanisms of oxygen metabolism, Adanc. Enzymol., 19: 79-233.
- May, S. W. and Abbott, B. J. 1973. Enzymatic epoxidation. The Journal of Biological Chemistry, 248: 1725-1730.
- Matthews, R. F. and Braddock, R. J. 1987. Recovery and applications of essential oils from oranges. Food Technol. 41 (1):57-61.
- Mattiason, B. 1983. Applications of aqueous two-phase systems in biotechnology. Trends in Biotechnology. 1: 16-20.
- Mikami, Y., Watanabe, E., Fukunaga, Y., Kisaki, T. 1978. Formation of 2S-hydroxy- β -ionone and 4R-hydroxy- β -ionone by microbial hydroxylation of β -ionone. Agric. Biol. Chem. 42: 1075-1077.
- Moddcox, I.S., Dunhill, P. and Lilly, M.D., 1981. Use of immobilized cells of *Rhizopus nigricans* for the 11 α -hydroxylation of progesterone. Biotechnol. Bioeng., 23: 345-354.
- Mukherjee, B.B., Kraidman, G.K., and Hill, J.D. 1973. Synthesis of glycols by microbial transformation of some monocyclic terpenes. Appl. Microbiol. 25: 447-453.
- Narushima, H., Omori, T., and Midoda, Y. 1982. Microbial transformation of α -pinene. Eur. J. Appl. Microbiol. Biotechnol. 16: 174-178.
- Noma, Y., Yamsaki, S., and Asakawa, Y., 1992. Biotransformation of limonene and related compounds by *Aspergillus cellulosa*. Phytochemistry, 31 (8): 2725-2727.
- Ohlson, S., Flygare, S., Larsson, P.-O., and Mosbach, K. 1980. Steroid hydroxylation using immobilized spores of *Curvularia lunata* germinated in situ. J. Appl. Microbiol. Biotechnol. 10: 1-9.
- Omata, T., Tanaka, A., Yamane, T., and Fukui, S., 1979. Immobilization of microbial cells and enzymes with hydrophobic photo-crosslinkable resin prepolymers. European J. Appl. Microbiol. Biotechnol., 6: 207-215.

- Orrenius, S. and Ernster, L. 1974. Microsomal cytochrome P-450-linked mono-oxygenase systems in mammalian tissues. *Molecular Mechanisms of Oxygen Activation*, Osamu Hayaishi (Ed), Academic Press, pp 215-244.
- Ortiz de Montellano, P. R., Ed. 1986. *Cytochrome P-450: structure, Mechanism and biochemistry*. Plenum: New York.
- Osborne, S.J., Leaver, J., and Turner, M.K. 1992. Membrane concentrations of primary alcohols which inhibit progesterone 11 α -hydroxylase in *Rhizopus nigricans*, In: *Biocatalysis in Non-conventional Media*. Tramper, J., Vermue, M.H., Beeftink, H.H., and Von Stockar, U., Eds. Elsevier, London, pp. 31-36.
- Osborne, S.J., Leaver, J., Turner, M.K., and Dunnill, P. 1989. Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane. *Enzyme Microb. Technol.*, 12: 281-291.
- Ouazzani, J., Arseniyadis, S., Manzaneda, R. A., Rumero, A. and Ourisson, G. 1991. Microbial hydroxylations of further octalin derivatives. *Tetrahedron Letters*, 32 (17): 1983-1986.
- Peterson, J.A. and Coon, M.J. 1968. Enzymatic ω -oxidation, *J. Biol. Chem.*, 248: 329-334.
- Petzoldt, K. und Wiechert, R., 1976. Verfahren zur Herstellung von Ostren-3,17-dion-Derivaten. DE OS 24 56 068, Int. Cl. C 07 J 1/00, appl. 23. 11. 1974, publ. 12.8. 1976.
- Pinder, A.R. 1960. The essential oil. In "The chemistry of the terpenes", John Wiley & Sons Inc. New York. pp 106-131.
- Pinheiro, H.M., and Cabral, J.M.S. 1992. Screening of whole-cell immobilization procedures for the Δ^1 -dehydrogenation of steroids in organic medium. *Enzyme Microb. Technol.*, 14: 619-624.
- Prema, B.R. and Bhattacharyya, 1962. Microbial transformation of terpenes II. Transformations of α -pinene. *Appl. Microbiol.* 10: 524-528.
- Rosazza, J.P., 1982. *Microbial transformations of bioactive compounds*. CRC Press, Inc., Boca Raton, Florida, pp. 45-107.
- Rosenberg, M., Gutnick, D., and Rosenberg, E. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.*, 9: 29-33.

- Salter, G.J., and Kell, D.B. 1995. Solvent selection for whole cell biotransformations in organic media. *Critical Reviews in Biotechnology*, 15 (2): 139-177.
- Schlosser, D. and Schmauder, H.-P. 1991. 15 α -Hydroxylation of 13-ethyl-gon-4-ene-3,17-dione using a hyphal fungus immobilized in calcium alginate gel beads. *J. Basic Microbiol.* 31 (5): 385-390.
- Schlosser, D., Irrgang, S., and Schmauder, H.-P. 1993. Steroid hydroxylation with free and immobilized cells of *Penicillium raistrickii* in the presence of β -cyclodextrin. *Appl Microbiol Biotechnol*, 39: 16-20.
- Schmauder, H., P., Schlosser, D., Gunther, T., Hattenbach, A., Sauerstein, J., Jungnickel, F., and Augsten, H., 1991, Application of immobilized cells for biotransformations of steroids, *J. Basic Microbiology*, 31(6): 453-477.
- Schreier, P. 1989. Aspects of biotechnological production of food flavors, *Food Reviews International*, 5(3): 289-315.
- Schunck, W.-H, Kargel, E., Gross, B., Wiedmann, B., Mauersberger, S., Kopke, K., Kiebling, U., Strauss, M., Gaestel, M., and Muller, H.-G. 1989. Molecular cloning and characterization of the primary structure of the alkane hydroxylating cytochrome P-450 from the yeast *Candida maltosa*. *Biochem. Biophys. Res. Commun.* 161: 843-850.
- Shteinman, A.A., 1995, The mechanism of methane and dioxygen activation in the catalytic cycle of methane monooxygenase, *FEBS Letters*, 362: 5-9.
- Shukla, O.P., Moholay, M.N., and Bhattachacharyya, A.P.K. 1968. Microbiological transformations of terpenes: Part X- Fermentation of α - and β -pinene by a soil pseudomonad (PL-strain). *Indian J. Biochem.* 5: 79-91.
- Shulka, O.P. and Bhattacharyya, P.K. 1968. Microbiological transformations of terpenes: Part XI-Pathway of degradations of α - and β -pinene by a soil pseudomonad (PL-strain). *Indian J. Biochem.* 5: 92-101.
- Smith, M., Zahnley, J., Pfeifer, D., and Goff, D., 1993. Growth and cholesterol oxidation by *Mycobacterium species* in tween 80 medium. *Applied and Environmental Microbiology*, 59 (5): 1425-1429.
- Sode, K., Karube, I., Araki, R., and Mikami, Y. 1989. Microbial conversion of β -ionone by immobilized *Aspergillus niger* in the presence of an organic solvent. *Biotechnol. Bioeng.* 33: 1191-1195.

- Sonomoto, K., Nomura, K., Tanaka, A., and Fukui, S. 1982. 11α -hydroxylation of progesterone by gel-entrapped living *Rhizopus stolonifer* mycelia. Eur. J. Appl. Microbiol. Biotechnol., 16: 57-62.
- Sonsbeek, H.M.V., Beeftink, H.H., and Tramper, J. 1993. Two-liquid-phase bioreactors. Enzyme Microb. Technol., 15: 722-729.
- Sprecher, E. and Hanssen, H.-P. 1985. Recent trends in the research on flavor produced by fungi. Topics in Flavour Research. pp 387-403.
- Steinert, H.-J., Vorlop, K.D., and Klein, J. 1987. Steroid side chain cleavage with immobilized living cells in organic solvents, In: Biocatalysis in Organic Media. Lanne, C., Tramper, J., and Lilly, M.D., Eds., Elsevier, Amsterdam, pp 51-63.
- Stumpf, B., Abraham W-R, Kieslich, K., 1983. Verfahren zur Herstellung von (+)- α -Terpineol durch mikrobiologische Umwandlung von Limonen. German Patent P 3243090.
- Stumpf, B., Abraham, W.R., Kieslich, K. 1982. Germ. Pat. Appl. 3 243 091. Verfahren zur herstellung von (R)-4-hydroxy- β -ionon durch mikrobiologische umwandlung von β -ionon.
- Stumpf, B., Abraham, W.R., Kieslich, K., 1982. Germ. Pat. Appl. 3 243 090. Verfahren zur herstellung von (+)- α -terpineol durch mikrobiologische umwandlung von limonen.
- Suhara, Y., Itoh, S., Ogawa, M., Yokose, K., Sawada, T., Sano, T., Ninomiya, R., and Maruyama, H.B. 1981. Regio-selective 10-hydroxylation of patchoulol, a sesquiterpene by *Pithomyces* species. Appl. Environm. Microbiol. 42: 187-191.
- Taniguchi, H., Imai, Y., and Sato, R. 1984. Substrate binding site of microsomal cytochrome P-450 directly faces membrane lipids. Biochem. Biophys. Res. Comm. 118: 916-922.
- Tonge, G. M., Harrison, D.E., and Higgins, I.J. 1977. Purification and properties of the methane mono-oxygenase enzyme system from *Methylosinus trichosporium* OB3B, Biochem. J. 161: 333-344.
- Trudgill, P. W. 1984. Microbial degradation of the alicyclic ring structural relationships and metabolic pathways. Microbial Degradation of Organic Compounds. Gibson, D.T. (Ed). Marcel Deller, Inc. pp 131-180.
- Tsai, K.P. 1984. Conversion of cellobiose to glucose using immobilized cell of *Aspergillus terreus*. M.S. Thesis, Louisiana State University.

- Tudroszen, N.J., Kelly, D.P., and Millis, N.F. 1977. α -Pinene metabolism by *Pseudomonas putida*. *Biochem. J.* 168: 315-318.
- Vermue, M., Sikkema, J., Verheul, A., Bakker, R., and Tramper, J. 1993. Toxicity of homologous series of organic solvents for the Gram-positive bacteria *Arthrobacter* and *Nocardia* sp. and the Gram-negative *Acinetobacter* and *Pseudomonas* sp. *Biotechnol. Bioeng.*, 42: 747-758.
- Vidyarthi, A.S. and Nagar, S.J. 1994. Immobilization fungal spores for microbial transformation of steroids: 11 α -hydroxylation of progesterone. *Biol. Memoirs*, 20 (1): 15-19.
- Weber, L., Dorre, M., Repp, H.-D., Stottmeister, U. and Haufe, G. 1988. Mikrobielle oxydation von α -pinen mit *Acetobacter methanolicus*. *Z. Chem.* 28: 98-99.
- Welsh, F.W., Murray, W.D., and Williams, R.E., 1989. Microbiological and enzymatic production of flavor and fragrance chemicals, *Critical Reviews in Biotechnology*, 9 (2): 105-169
- Whittaker, D., 1972. The monoterpenes, *Chemistry of Terpenes and Terpenoids*, Newman, A.A (Ed.), Academic Press, London and New York, pp 11-87.
- Wulf, O.D., and Thonart, P. 1989. Bioconversion of vanillin to vanillyl alcohol in a two-phase reactor. I. Parameters improvement. *Applied Biochemistry and Biotechnology*, 20/21: 165-180.
- Yamane, T., Nakatani, H., and Sada, E., 1979. Sterid bioconversion in water-insoluble organic solvents: Δ^1 -Dehydrogenation by free microbial cells and by cells entrapped in hydrophilic or lipophilic gels. *Biotechnology and Bioengineering*, 21: 2133-2145.
- Yang, S.K. 1988. Stereoselectivity of cytochrome P-450 isozymes and epoxide hydrolase in the metabolism of polycyclic aromatic hydrocarbons. *Biochemical Pharmacology*, 37 (1): 61-70.
- Yoo, S., 1993. Bioconversion of α - and β -pinene. M.S. Thesis, Louisiana State University.
- Zimmer, T., Kaminski, K., Scheller, U., Vogel, F., and Schunck, W.-H. 1995. In Vivo reconstitution of highly active *Candida maltosa* cytochrome P450 monooxygenase system in inducible membranes of *Saccharomyces cerevisiae*. *DNA and Cell Biology*, 14 (7): 619-628.

VITA

The author was born in Sichuan Province, People's Republic of China, on March 14, 1964. He graduated from Southwest Agricultural University in People's Republic of China with a bachelor of science degree in Agronomy in August 1984. After graduation from the University, he received a job from the Chinese Academy of Sciences in Beijing, China, as a research scientist in the Commission for Integrated Survey of Natural Resources for 6 years. Major work during that period of time was involved in studying the agro-ecological systems and environmental protection in rural areas in China.

The author was accepted as a graduate student in the Agronomy Department of Louisiana State University in 1990. He obtained his master of science degree from the Department in 1992. Since 1993, he has continued his study in the Food Science Department of Louisiana State University as a candidate for the degree of Doctor of Philosophy.

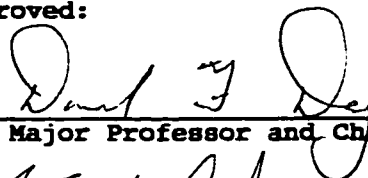
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Qiang Tan

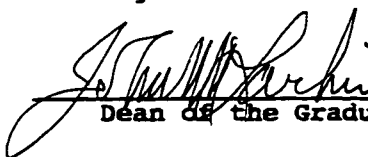
Major Field: Food Science

Title of Dissertation: IMMOBILIZATION OF P.DIGITATUM AND BIOCONVERSION
OF LIMONENE TO ALPHA-TERPINEOL

Approved:

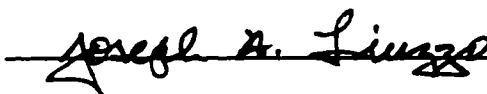


Major Professor and Chairman

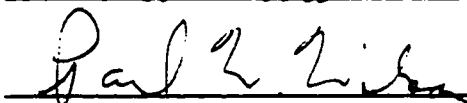


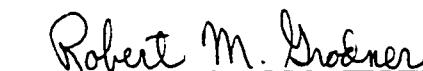
Dean of the Graduate School

EXAMINING COMMITTEE:









Date of Examination:

7/24/1996